

Biofuels Program Semiannual Report

Second Half FY 2000

R. Wooley



NREL

National Renewable Energy Laboratory

1617 Cole Boulevard
Golden, Colorado 80401-3393

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Prepared under Task No. BFP1A101



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Table of Contents

Pretreatment Technology	1
Summary of Technical Achievements or Results	1
General Technical or Scientific Progress	1
Scientific Publications, Presentations, and Other Activities	5
Improved Cellulase Enzymes	7
Summary of Technical Achievements or Results	7
General Technical/Scientific Progress	8
Scientific Publications, Posters, Presentations, and other Activities	9
Strain Development	13
Summary of Technical Achievements or Results	13
General Technical or Scientific Progress	14
Scientific Publications, Presentations, and Other Activities	17
Enzymatic Process Development	18
Summary of Technical Achievements or Results	18
General Technical/Scientific Progress	18
Scientific Publications, Presentations, and Other Activities	25
Lignin Conversion to Fuels	27
Summary of Technical Achievements or Results	27
General Technical/Scientific Progress	28
Scientific Publications, Presentations, and Other Activities	30
Collaborative Industrial Process Development	31
Summary of Technical Achievements or Results	31
General Technical/Scientific Progress	32
Scientific Publications, Presentations, and Other Activities	34
Process Engineering And Development.....	36
Summary of Technical Achievements or Results	36
General Technical and Scientific Progress	36
Scientific Publications, Presentations, and Other Activities	39
Renewable Diesel Project	40
Summary of Technical Achievements or Results	40
General Technical/Scientific Progress	40
Scientific Publications, Presentations and, Other Activities	43

PRETREATMENT TECHNOLOGY

Summary of Technical Achievements or Results

Work by the Pretreatment Technology Team during this reporting period was focused in four primary areas: 1) Operation of the engineering-scale countercurrent pretreatment reactor system and reporting of initial process performance data; 2) Organizing and conducting an external peer review meeting on the status and future direction of the engineering-scale countercurrent pretreatment reactor project; 3) Continued initial investigation leading to a more fundamental understanding of hydrolysis chemistry mechanisms, potentially leading to the identification of less severe, less complex, and/or less expensive thermochemical pretreatment/hydrolysis reaction conditions and reactor designs; and 4) Utilizing our current knowledge and the current programmatic direction to make a decision on the future status of the countercurrent pretreatment reactor system project.

General Technical or Scientific Progress

Operation of the Engineering-Scale Countercurrent Pretreatment Reactor System and Reporting of Initial Process Performance Data

The countercurrent pretreatment reactor system was operated in a fully integrated manner at targeted operating conditions for a total of more than 100 hours. Twenty-two steady-state mass balance runs have been conducted, with eighteen runs having chemical compositional data available for mass balance and yield calculations. Of those eighteen runs, thirteen met the selection criteria of having both carbon and key component (glucose, xylose, lignin) mass balance closures of $100\% \pm 25\%$. The overall carbon balance for these runs is $96.1\% \pm 11.5\%$.

Two of the three initial performance targets were substantially met, while one was not met. The soluble xylose yield average for the thirteen selected runs is $82.8\% \pm 17.6\%$, compared to the target of 85%. The soluble glucose yield average for the thirteen selected runs is $17.1\% \pm 4.1\%$, with an unconverted cellulose recovery of $75.2\% \pm 11.3\%$. Performance targets are 60% glucose yield with 25% recovery of unconverted cellulose. The total soluble sugar concentration for all thirteen runs is 42.9 g/L, with the five highest concentration runs averaging 58.0 g/L and the highest concentration run achieving 66.5 g/L. The milestone target is 60 g/L.

The glucose yield target was not met, although the shortfall was almost completely attributable to unconverted cellulose, with $< 5\%$ unaccounted for or lost to degradation products. The main reason for the inability to achieve the glucose yield target has been attributed to the inability to operate the vertical stage of the reactor system in a “shrinking-bed” mode, which is necessary to overcome the hypothesized boundary layer mass transfer resistances needed to achieve high glucose yields at the relatively mild reaction conditions. In some cases, much of the cellulose is being transported out of the reactor system as fine suspended particles in liquid streams before these particles can reach or be exposed to the required residence times in the vertical stage of the reactor system. In

other cases, the bulk of the cellulose particles are passing through the entire reactor system, but the solid-liquid contacting required to disrupt the hypothesized boundary layer is insufficient. In any event, modifications are needed to allow the vertical stage of the reactor system to operate in a shrinking-bed mode. One modification involves increasing the fibrous nature of the solids in the vertical stage, either by using larger feedstock particles, limiting the extent of reaction in the horizontal stage, or reducing the mechanical grinding of the feedstock that is occurring prior to the vertical stage. Another modification involves redesigning the vertical stage screw conveyor to result in less mixing and a more packed bed of solids.

Further details on the operational data obtained from the engineering-scale countercurrent pretreatment reactor system have been reported in FY 2000 Biofuels Program C Milestone 11 (Process Performance Data from Countercurrent Pretreatment Reactor System).

Organizing and Conducting an External Peer Review for the Engineering-Scale Countercurrent Pretreatment Reactor Project

Concerns about the practicality of the shrinking-bed countercurrent pretreatment approach were raised in Biofuels Program external peer review meetings in January, 1998 and July, 1999. In the time since these meetings, much of our work has been aimed at addressing these concerns. In August 2000, an external peer meeting was held. This meeting was focused specifically on the countercurrent pretreatment reactor project. The review panel consisted of:

Davis Clements, Professor, University of Nebraska - Lincoln
Alvin Converse, Professor Emeritus, Dartmouth College
Raphael Katzen, Consulting Engineer, PK-PE
Keith Mackie, Chief Science Advisor, New Zealand Forest Research Institute Limited

These gentlemen all have significant backgrounds in the pretreatment of biomass. Dr. Clements and Dr. Katzen served on previous programmatic review meetings and were foremost in raising the process operability concerns. Dr. Joe Glas of BC International had also agreed to serve on this review panel, but was unable to attend. Other external meeting participants who provided technical information to the review panel were: Anders Mokvist and Chris Kajzer of Valmet Inc., Lynn Montague and Carol Babb of Harris Group Inc., and John Brady of Cornell University.

The primary purpose of the review meeting was to evaluate the results that had been achieved during operation of the engineering scale reactor system and to determine whether the operability concerns had been satisfactorily addressed. The review panel offered four primary recommendations. These recommendations are listed below:

1. The shrinking-bed experiments have been an important development. Determine how much can be explained with homogeneous kinetics.
 - Then, if needed, investigate transport phenomenon by experimental means. Document in peer reviewed literature.
2. First Stage Reactor
 - Develop Conditions to:
 - ❖ Minimize furfural production and lignin solubilization.
 - ❖ Maximize hemicellulose removal, and improve the physical properties of the solids for the second stage. This probably will require lower temperatures and more acid.
3. Second Stage Reactor
 - Maximize glucose and minimize hydroxyl-methyl-furfural (HMF) production.
 - Try to achieve the performance expected in a shrinking-bed countercurrent reactor.
 - Try operating without the screw in plug flow, at a higher L/D ratio, and with some experiments using softwood.
 - Minimize dissolved lignin. This will also probably require lower temperatures and more acid.
4. After reactor objectives have been met, reevaluate the acid hydrolysis process.

Our interpretation and response to these specific recommendations are discussed in detail in FY 2000 Biofuels Program C Milestone 17 (Reach Go/No Go Decision on the Feasibility of the Counter-current Pretreatment Reactor Project Using the Minimum-scale Engineering Reactor).

Fundamental Understanding of Cellulose and Hemicellulose Hydrolysis Mechanisms

In an attempt to mechanistically explain the high yields achieved in the bench scale shrinking-bed apparatus, we are continuing to develop a more fundamental understanding of the hydrolysis chemistry mechanisms involved. A hypothesis based upon a heterogeneous reaction mechanism and the requirement to disrupt a water-molecule boundary layer has been developed to explain the superior yields achieved in the present configuration of the countercurrent pretreatment process. If this hypothesis is correct, there may be other chemical methods for disrupting this boundary layer besides the scouring effect of the flowing countercurrent, shrinking-bed process. This may allow both hemicellulose and cellulose hydrolysis at lower temperatures and pressures. Preliminary

molecular modeling work by J. Brady (Cornell University) has identified the existence of a highly ordered water-molecule boundary layer at the surface of cellulose. Work is continuing to determine the true nature of this boundary layer and whether chemicals (such as those derived from soluble lignin) could serve as a means to disrupt this layer. In addition, subcontracted work by Y.Y. Lee (Auburn University) has indicated that temperatures as low as 205°C with 0.07% flowing sulfuric acid in a bench-scale shrinking-bed reactor system can achieve xylose and glucose yields similar to those achieved at 225°C. Temperatures as low as 180°C with 0.07% sulfuric acid have been tested with 60% glucose yields and 95% xylose yields (as oligomers) in the shrinking-bed apparatus. These conditions are very mild for even a prehydrolysis reaction and indicate that reactor design considerations for prehydrolysis may potentially allow for significantly less severe (and less expensive) prehydrolysis processes.

Decision of the Future Status of the Countercurrent Pretreatment Reactor System Project

Based upon our current knowledge of technical and strategic issues, a decision has been made regarding the future of the countercurrent pretreatment reactor project. That decision is to effectively discontinue development of the shrinking-bed total hydrolysis concept in the engineering scale reactor system. The engineering scale reactor system has been operated for one initial campaign of runs, meeting some of the initial process performance objectives while not meeting others. Several recommended mechanical and operational changes have been developed in conjunction with the equipment supplier and an external panel of pretreatment technology experts. Many of these changes will be implemented, but within the context of expanding the capabilities of the engineering scale reactor system as a flexible device for development and testing of improved prehydrolysis concepts that mesh with the expected improved cellulase enzyme technology.

The development of the countercurrent shrinking-bed process has led to new insights regarding the impact of reaction configuration of thermochemical hemicellulose and cellulose hydrolysis. Hydrolysis yields have been achieved in bench scale systems that are higher than previously thought possible, especially as related to glucose yields from cellulose. The fundamental mechanisms regarding hemicellulose and cellulose hydrolysis performance with respect to different reaction configurations are still not fully understood. We will continue to pursue these new reaction concepts through fundamental modeling and experimental work. This will ultimately lead to the most effective reactor designs in terms of process performance, reactor configuration and operability, and cost.

Scientific Publications, Presentations, and Other Activities

General Presentations/Travel

R. Torget met with Dr. John Brady and students and gave a seminar at Cornell University, Ithaca, NY on April 11, 2000.

R. Torget met with professors and gave a seminar at the Thayer School of Engineering, Dartmouth College, Hanover, NH on April 13, 2000.

R. Elander, R. Wooley, and A. Aden met with Harris Group, Inc. in Seattle, WA on April 26, 2000 to discuss a modification to their subcontract to address specific reactor design issues for the countercurrent pretreatment reactor project.

R. Torget attended a meeting of the Biomass Refining Consortium for Applied Fundamentals and Innovation in Gatlinburg, TN on May 10, 2000.

R. Elander met with E. Lehrburger and R. Wingerson of PureVision Technology, Inc. on May 24, 2000 to discuss advanced pretreatment process technologies.

R. Elander, R. Torget, and N. Nagle gave a presentation to and reviewed the status of the countercurrent pretreatment reactor system with A. Mokvist and C. Kajzer of Valmet Corp. (formerly Sunds Defibrator Inc.) on July 24-25, 2000.

R. Elander, R. Torget, and B. Hames met with Mark Paster of the Department of Energy (DOE) Office of Industrial Technologies (OIT) on July 25, 2000 to discuss the countercurrent pretreatment project and other advanced pretreatment and fractionation processes.

R. Elander and G. Santos-Leon (DOE Office of Fuels Development) met with T. Werpy and others at the Pacific Northwest National Laboratory (PNNL) in Richland, WA on August 29, 2000 to discuss NREL's potential role in the ADM/NCGA/PNNL corn fiber refining project.

R. Elander, M. Yancey, and J. Ferrell (DOE Office of Fuels Development) met with Dr. Thomas Hanley and other professors and students at the University of Louisville in Louisville, KY on September 6, 2000 to develop work activities for an upcoming subcontract.

Scientific Meetings: Papers and Posters Presented or Recently Accepted for Presentation

A poster presentation entitled "Dilute Sulfuric Acid Fractionation of Hardwood—A Novel Pilot Scale Reactor Design" by R. Torget, R. Elander, A. Haverkamp, D. Johnson, and B. Hames was given at the conference entitled Wood and Cellulose: Building Blocks for Chemicals, Fuels, and Advanced Materials, April 9-11, 2000, Syracuse University, Syracuse, NY.

The following presentations were given at the 22nd Symposium on Biotechnology for Fuels and Chemicals in Gatlinburg, TN, May 7-11, 2000:

A poster presentation, "Kinetics Aspects of the Dilute-Acid Catalysis and Autohydrolysis of Hardwood Hemicellulose" by R. Torget, A. Haverkamp, and B. Hames.

A poster presentation, "Kinetics of Cellulose Hydrolysis under Extremely Low Sulfuric Acid and High Energy Conditions" by J.S. Kim (Auburn University), Y.Y. Lee (Auburn University), and R. Torget.

A poster presentation, "Investigation of Hot Separation and Washing Techniques to Produce Highly Digestible Pretreated Solids Following Dilute Acid Prehydrolysis of Hardwood" by R. Elander and R. Torget.

Scientific Journals: Papers Accepted for Publication

A paper entitled "Fundamental Aspects of Dilute-Acid Hydrolysis/Fractionation Kinetics of Hardwood Carbohydrates - Part I: Cellulose Hydrolysis" by R. Torget, J.S. Kim (Auburn University) and Y.Y. Lee (Auburn University) was published in *Industrial & Engineering Chemistry Research*, 39, 2817-2825 (2000).

Project Operation

The Process Engineering Team subcontract entitled "Process Design and Cost Estimation of Critical Equipment in the Biomass to Ethanol Process" being performed by Harris Group Inc. (Lynn Montague, Principal Investigator) was modified with an extension through September 30, 2000 at an added cost of \$42,696 from Pretreatment task funds to address specific issues related to the countercurrent pretreatment reactor design process operability issues.

The subcontract entitled "Lignin Recovery and Upgrading, Kinetic Modeling" being performed by Auburn University (Dr. Y.Y. Lee, Principal Investigator) was modified with an extension through September 30, 2000 at an added cost of \$10,000.

IMPROVED CELLULASE ENZYMES

Summary of Technical Achievements or Results

- Cellulase Synergism. Understanding the roles and relationships of cellulase component enzymes acting on specific substrates is vital to the development of an efficient artificial cellulase system for the conversion of cellulosic biomass to sugars. The *Trichoderma reesei* biomass degrading system consists of many glycosyl hydrolases, of which five β -1,4-endoglucanases, two β -1,4-exoglucanases, two xylanases, a β -D-glucosidase, an α -L-arabinofuranosidase, an acetyl xylan esterase, a β -mannanase, and a α -glucuronidase have been sequenced.

Application of preliminary Proteomics analysis to a standard commercial cellulase product was judged appropriate for demonstrating fingerprinting methodology. Although 2-D protein gels have been used on occasion to follow the expression of selected *T. reesei* cellulase components and their glycosylated forms, a systematic display of the entire system of enzymes found in *T. reesei* culture broth has not been reported. We conducted the fingerprinting via 2-D gel electrophoresis and internal peptide sequence analysis of a commercial cellulase preparation, Spezyme from Genencor International. The Biomolecular Research Facility at the University of Virginia Medical School processed gels prepared at NREL for spot sequencing and identification. Forty-five spots were identified by MALDI-TOF Mass Spectrometry. The experimental results were compared to ~1100 known glycosyl hydrolases from all species, with positive hits only arising from *T. reesei* proteins. Most of these were of known proteins, but several novel proteins were detected. A surprisingly high percentage of the cellulases identified were fragmented and otherwise modified (i.e., a wide range of megawatt (MW) and charge heterogeneity for all cellulases was detected).

- Cellobiohydrolase (CBH) Improvement. In terms of its structural stability as determined by differential scanning microcalorimetry and its enzymatic performance as measured in the dialysis saccharification assay (DSA) on the substrate pretreated yellow poplar, *P. chrysosporium* CBH58 is virtually indistinguishable from *T. reesei* CBH I. Both have T_{\max} values near 64.5°C. When each CBH is evaluated in terms of saccharification of PYP (in a 19:1 mixture with *A. cellulolyticus* E1cd), the DSA progress curves for the two cellobiohydrolases are superimposable both at 0°C and at 60°C. At 60°C, the thermal inactivation during the assay is enough of a factor to decrease the 120-h yield of the *T. reesei*-CBH I-catalyzed reaction by 37%. Both CBHs show increased initial velocities at 60°C as compared to the velocities at 40°C, and both enzymes have their ultimate yields substantially reduced by the effect of thermal inactivation during the assay. CBH58 is thus seen to be neither more nor less thermally stable than *T. reesei* CBH I. The fact that CBH58 is just as effective as *T. reesei* CBH I in the saccharification of the test lignocellulosic substrate, however, makes it a valuable subject for further study.
- The automated filter paper assay based on a highly modified Cyberlab C-400 robotics deck has passed all mechanical and electronic operational criteria. In order to conduct

an automated version of the 1987 Ghose FP assay (the international standard), an off-the-shelf C-400 deck was equipped with a foil-type plate sealer, a cooling shelf for reagents, a bar code reader, three custom 60°C and 95°C ovens, and a special plate foil punching system. The deck is now being validated for the FPU test against values obtained manually.

- **Cellulase Modeling.** Designing improved versions of cellulases would be facilitated by both a quantitative and qualitative understanding of the role of various interactions in substrate binding. Molecular dynamics simulations conducted at Cornell University employing umbrella sampling techniques have been used to calculate the potential of mean force for the binding of a beta-D-glucopyranoside molecule to a benzene molecule in aqueous solution as a model for the binding of sugar substrates to phenylalanine residue in proteins. The interaction of these two molecules was strongly affected by hydration, as expected, with their nonpolar faces pairing by hydrophobic association to minimize the exposure of apolar groups to water. This experience with small molecules is being used to design more extensive models incorporating larger protein and substrate domains which should result in gaining critical understanding of cellulase action. NREL's recent acquisition of a new SGI Octane SE computer running CHARMM will greatly facilitate the ongoing project collaboration between NREL and Cornell University.
- **Reducing Cellobiose Inhibition.** We are investigating a new active site amino acid modification recently discovered during a routine examination of *A. cellulolyticus* EI. Site N204 appears to pose leaving group stabilization and its mutation to alanine is expected to further reduce end product inhibition in EI, perhaps to near the theoretical limit. We have constructed and purified the N204A EI mutant and are currently testing its kinetic properties in DSA as well as closed tube assays.

General Technical/Scientific Progress

Site-Directed-Mutagenesis of *T. reesei* CBH I

Trichoderma reesei CBH I is a mesophilic cellobiohydrolase which plays a major role in the hydrolysis of microcrystalline cellulose. To enzymatically hydrolyze biomass more effectively, it is essential that key thermophilic cellulases are developed or discovered.

The objective of this research was to develop host expression systems for *T. reesei* CBH I suitable for high-throughput screening (HTS). Host expression systems for HTS must form discrete, small colonies, have high transformation efficiencies, and produce active, biologically relevant enzymes. *E. coli* exhibits many characteristics desirable for HTS, however, *T. reesei* CBH I has been historically difficult to express in an active, soluble form from bacteria. This is due principally to the well established observation that proteins containing multiple disulfide bonds critical to protein conformation are extremely difficult to express from prokaryotic hosts in active forms. *T. reesei* has ten disulfides and one unpaired cysteine in the cd. To address this problem, we identified and tested new host strains of *E. coli* that were specifically designed to assist disulfide bond formation and permit the production of active enzymes from heterologous genes (especially genes from

higher organisms). We found that only the catalytic domain (cd) of CBH I can be expressed at high levels from the K-12 Origami™ strain of *E. coli* with some success. Although soluble enzyme yields are low and the enzyme is difficult to purify, we are hopeful that following further modifications to both the host and enzyme coding sequences, the *E. coli* based expression systems will prove effective for HTS of CBH Icd.

Scientific Publications, Posters, Presentations, and other Activities

General Presentations

“Improved Cellulases for Bioethanol Production,” W.S. Adney, J.O. Baker, S.R. Decker, S.L. McCarter, J. Sheehan, T.B. Vinzant, and M.E. Himmel, *Wood and Cellulose: Building Blocks for Chemicals, Fuels, and Advanced Materials*, SUNY College of Environmental Science & Forestry, Syracuse, NY, 4/9/00-4/11/00.

“Site-Directed Mutagenesis of the EI Endoglucanase from *Acidothermus cellulolyticus*,” S.L. McCarter, W.S. Adney, J.O. Baker, T.B. Vinzant, R.D. Guckian, S.R. Decker, J. Sakon, and M.E. Himmel. The 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN. May, 2000.

“Heterologous Expression of *Trichoderma reesei* CBH I: Effect of Site-Directed Mutations on Expression and Thermostability,” S.R. Decker, W.S. Adney, J.O. Baker, S.L. McCarter, T.B. Vinzant, J. Sakon, K.L. Barnett, and M.E. Himmel. The 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, May, 2000.

Patent Activities

Notice that MRI has approved the “Cellulase Animation” video for copyright protection was received. Both the full-length, 8-minute “academic” version and a new 5-minute “executive” version of the video are available.

Scientific Journals and Books

“Investigation of the Cell Wall Loosening Protein Expansin as a Possible Additive in the Enzymatic Saccharification of Lignocellulosic Biomass,” J.O. Baker, M.R. King, W.S. Adney, S.R. Decker, T.B. Vinzant, S.L. Lantz, R.E. Nieves, S.R. Thomas, L.C. Li, D.J.

Cosgrove, and M.E. Himmel. Applied Biochemistry and Biotechnology, (84-86), pp. 217-223, (2000).

"Two Novel Alkalotolerant Dextranases from *Streptomyces anulatus*," S.R.. Decker, W.S.. Adney, T.B. Vinzant, and M.E. Himmel, In *Glycosyl Hydrolases for Biomass Conversion*; (M. Himmel, J.O. Baker, and J. Saddler, eds.), ACS Series 769, Chapter 13, American Chemical Society: Washington, DC, 2000, pp. 222-235.

"Molecular Mechanics Studies of Cellulases," R. Palma, M.E. Himmel, G. Liang, and J. Brady. In *Glycosyl Hydrolases for Biomass Conversion*; (M.E. Himmel, J.O. Baker, and J. Saddler, eds.), ACS Series 769, Chapter 7, American Chemical Society: Washington, DC, 2000, pp. 112-130.

"Calculation of the Potential of Mean Force for the Binding of Glucose to Benzene in Aqueous Solution," R. Palma, M.E. Himmel, and J. Brady, J. Phys. Chem. B 104, pp. 1228-7234, (2000).

"Glycosyl Hydrolases for Biomass Conversion," (M.E. Himmel, J.O. Baker, and J. Saddler, eds.), ACS Book Series 769. American Chemical Society: Washington, DC, (2000).

"Fingerprinting a Commercial *Trichoderma reesei* Cellulase Preparation," T.B. Vinzant, W.S. Adney, S.R. Decker, J.O. Baker, M.T. Kinter, N.E. Sherman, J.W. Fox, and M.E. Himmel, Appl. Biochem. Biotechnol. 2000, In Press.

Technology Transfer

M. Himmel was invited to give a presentation entitled "Improved Cellulases for Bioethanol Production," at the Biotechnology Center, University of Utah, Logan, UT, March, 2000.

M. Himmel was invited to present an update of NREL's cellulase research at Genencor International in Palo Alto, CA, July, 2000.

B. Adney attended an IBC sponsored conference on Proteomics for Rapid Target Identification and Bioinformatics for Genome Analysis and Function in Tucson, AZ, October 16-19, 2000. Topics of the conference were wide-ranging but focused primarily on high-throughput proteomics platforms for discovery of proteins and database hardware and software considerations in bioinformatics.

M. Himmel and J. Baker co-edited (with J. Saddler, University of B.C.) a new American Chemical Society (ACS) Book entitled "Glycosyl Hydrolases for Biomass Conversion." This 300-page book has been printed and is now available as ACS volume 769. The book features 14 chapters from internationally recognized researchers.

Project Operation

Subcontract (S/C): Design and Test Improved Assays for Endoglucanases and Exoglucanases. The subcontractor, the University of California at Davis, is evaluating cellulases for effectiveness on cellulose and/or representative lignocellulosic substrates under controlled experimental conditions. Standard methods are being developed for measuring cellulase activity on high molecular weight cotton cellulose and bacterial cellulose from *Acetobacter xylinum*, as well as on selected lignocellulosic materials. This work focuses on the release of soluble hydrolysis products as measured by the Bicinchoninic (BCA) method, and on changes in molecular-weight distribution of insoluble substrates as measured by the HPSEC-MALLS. The specific objective is to use both methods to contrast endo- and exo- mode of enzyme action. The proposed combinations of enzymes include *Trichoderma reesei* EGI/*T. reesei* CBH I and *Acidothermus cellulolyticus* E1/*T. reesei* CBH I. The HPSEC-MALLS method is being developed for analysis of cotton and bacterial cellulose using a DMAc/LiCl solvent system. Cellulose dissolved in LiCl/DMAc has been reported to be a true solution, to be stable over a long time, and to exhibit no degradation during the dissolution process.

S/C: Provide High Resolution X-ray Structures. The subcontractor at the University of Arkansas (J. Sakon) worked to support site-directed-mutation studies of *Acidothermus* EI and *T. reesei* CBH I. Crystals of CBH Icd were obtained and are ready for diffraction. One key EI mutant has also been crystallized. Also, See D. Wilson's S/C update.

S/C: Develop and Test Improved *T. fusca* Cellulases. The subcontractor at Cornell University, D. Wilson, has worked to *improve* *T. fusca* E3 and E5 by site directed mutagenesis. Modeling work on Cel6A carried out in J. Brady's lab at Cornell suggests that Arg78 may play a significant role in catalysis. To test this, we used site-directed mutagenesis to produce two new Cel6A mutant enzymes: one with Ala in position 78 and one with lysine. The purified enzymes had carboxymethyl cellulose (CMC) activities of 0.37% of wild-type (WT) for the Ala and 24% of WT for the Lys mutant enzymes. These results confirm that Arg78 is important, but not essential, for catalysis and that a positive change in this position increases activity over an unchanged residue. Wilson also mutated Ser84 and Ser85 to Ala, both individually and together. These mutations were prepared because a mechanism for *T. reesei* Cel6A was proposed in the literature in which the Ser equivalent to Ser85 was hydrogen bonded to the catalytic water molecule. All these mutant enzymes had near WT activity on CMC, so it is very unlikely that Ser85 is hydrogen bonded to the catalytic water. Wilson's lab is preparing selenocysteine labeled *T. fusca* Cel6Bcd for J. Sakon so he can crystallize it and determine the phases for completing an X-ray structure.

S/C: Model Cellulase/Cellulose Interactions. The subcontractor at Cornell University, J. Brady, has worked to apply molecular mechanics modeling to enhance our understanding of cellulase action. Experimental results suggest that there is a substantial conformational shift in structure of *T. fusca* E2 upon binding of the substrate. Various Molecular Mechanics simulations have been used to investigate possible alternate conformations of the protein bound to a tetrasaccharide substrate. Several alternate conformations of reasonable energy have been identified, including one in which the overall energy of the enzyme-substrate complex in solution is lower than that for the complex using the crystal

conformation. Based on the results of molecular dynamics simulations of the enzyme-substrate complex, a new mechanism of catalysis has been proposed.

Total Quality Management and ES&H

The Enzyme Technology (ET) team received NREL Waste Minimization training.

B. Adney continues to serve as Chairman of the NREL Biosafety panel.

STRAIN DEVELOPMENT

Summary of Technical Achievements or Results

A formal comprehensive research and development agreement (CRADA) contract was signed between NREL and Arkenol Holdings of Mission Viejo, California to develop a genetically altered microorganism capable of rapid and efficient fermentation of sugars derived from rice straw biomass to ethanol. The CRADA was set up after NREL identified a strain of *Zymomonas mobilis* that had superior fermentation performance in Arkenol hydrolysates. The one year agreement officially started on August 1, 1999.

The objective of this CRADA will be to develop superior strains of a patented bacterium that could lead to faster and more efficient fermentation of rice straw to ethanol. The *Z. mobilis* strain which has been identified as a good candidate for Arkenol's process will be genetically manipulated to create a stable strain capable of fermenting both six-carbon and five-carbon sugars. In addition to the genetic work, NREL will utilize its Process Development Unit (PDU) facility to generate large-scale fermentation data and materials useful for Arkenol acquiring engineering guarantees for their planned facility in Sacramento. Several experiments will be conducted to show the stability of the microorganism and the repeatability of the process.

The fermentation work that Arkenol requested was to conduct a five stage fill and draw fermentation at a minimum scale of 150 liter fermentation vessels. A fill and draw fermentation is a batch fermentation where at the completion of the fermentation, the majority of the culture is drawn from the tank. That volume is replaced with fresh media (or hydrolysate) and restarted using the portion of the previous culture (left in the tank) as the starting inoculum for the second batch. Arkenol requested that the fill and draw operation be repeated for five fermentations to determine the consistency and stability of the organism. They also asked that we repeat the entire 5 stage run in triplicate to verify the repeatability of the fermentation process.

The fermentations were conducted using a plasmid bearing *Zymomonas mobilis* strain capable of fermenting xylose and has shown to be tolerant to high sugar feedstocks. Arkenol does not have the capability to supply the quantities of hydrolysate needed for this scale of operation, so a synthetic form of hydrolysate was used for these experiments. The fermentations were conducted as soon as modifications were made to the vessels in order to obtain accurate carbon dioxide measurement and approval by the Institutional Biosafety Committee to use recombinant *Zymomonas* in the pilot plant.

Two runs were completed and showed very consistent and repeatable results. Final ethanol process yields for the five stages averaged 90.6% \pm 1.6% in the first run. For the second run, the average ethanol process yield was 89.1% \pm 0.9%. Arkenol was very pleased with the results from the duplicate runs that they did not request a third replicate be conducted.

General Technical or Scientific Progress

Develop Genomic Integrated Xylose-Fermenting *Z. Mobilis* Strain for High Sugar Fermentation

Two gene integration systems are being evaluated to determine which system effectively integrates xylose-fermenting genes into the chromosomal DNA of *Zymomonas mobilis* ATCC31821. We have started using homologous recombination methodology no integrants were formed using the *ldh* gene constructed from *Z. mobilis* ATCC39676 with a simple tetracycline marker. A longer fragment of the *ldh* gene was created by polymerase chain reaction (PCR) from 31821 and cloned into an appropriate vector containing a tetracycline (*Tc*) marker. An additional construct was made with the same *ldh* fragment flanking transaldolase/transketolase genes (half of the genes required for xylose fermenting capabilities) along with a *Tc* marker. After the genes are inserted into the cell, they are repeatedly cultured under selective conditions to allow recombination of genes to occur. During repeated transfers, the selection pressure is removed to select for non plasmid-bearing strains. After several weeks of transferring and plating on selective agar, we identified several potential isolates. A PCR screening experiment was conducted to determine the size of the *ldh* gene in the selected isolates. Ten isolates from each construct were evaluated and each produced a PCR product the size of the *ldh* fragment plus the additional *Tc* and *Tc/tal/tkt* genes. Southern hybridization indicated that the genes were integrated in the total genomic DNA. Further examination will be conducted to determine if the transaldolase and transketolase are functional.

Evaluation Of Fermentation Performance Of Integrated Xylose- And Arabinose-Fermenting *Z. Mobilis* Strains In Continuous Fermentation

Two genomic integrated xylose and arabinose-fermenting *Zymomonas* strains developed through both homologous recombination and transposition, AX101 and G8, and plasmid bearing strain 206C(pZB301) were evaluated for growth profile on pure sugar RMGXA (40:40:20 g/L for glucose, xylose and arabinose) at pH 5 and 5.5. At both pH, strain AX101 performed slightly better than the other two strains. At pH 5.5 the ethanol process yield was 83%, 84% and 76% for plasmid bearing strain, AX101 and G8 respectively. At pH 5 the ethanol yield was 83%, 83% and 67% respectively. Sugar utilization by strain AX101 was better than the other two strains at both pH. Both integrated strains had higher xylitol production than strain 206C(pZB301). Based on these results strain AX101 was selected for further evaluation in continuous fermentation.

A continuous fermentation using strain AX101 was started at pH was 5.5 and $T = 30^{\circ}\text{C}$. The media used was RMGXA (40:40:20 g/L). The starting dilution rate for both was 0.02 (1/h) equivalent to 50h residence time. The dilution rate was increased gradually after 5 cycles of steady state. At $D = 0.04$ (1/h) (25 h residence time) and the remaining sugar in the fermenter was 0, 1.6, and 5.6 g/L for glucose, xylose and arabinose, respectively, and the ethanol yield was 94%. After 28 days the pH was lowered to 5 and dilution rate was lowered to 0.027(1/h). The fermentation was continued for another 21 days at which time the dilution rate was 0.033 (1/h) and remaining sugar was 0, 1, and 6 g/L glucose, xylose and arabinose, respectively, and ethanol process yield is 90%. At this time the pH was

lowered further to 4.5 and fermentation was continued at dilution rate of 0.022 (1/h) then increased to 0.028 (1/h). The fermentation at pH 4.5 was stopped after 20 days at which time the remaining sugar and ethanol yield was similar to pH 5 results as mentioned above.

Construction of a DLDH- And LLDH-Negative *Lactobacillus* MONT4 Derivative

The construction of a DLDH- and LLDH-negative *L. MONT4* derivative utilizing multiple experimental approaches has been a quite challenging process. In order to better understanding the physiology of *L. MONT4* we have conducted experiments in which we followed growth, acid production, glucose utilization, lactic acid production, and ethanol production at several growth temperatures (37°C, 42°C, and 45°C) under both aerobic and anaerobic conditions. The specific growth rates of *L. MONT4* at 37°C, 42°C, or 45°C under anaerobic conditions are very similar. However, *L. MONT4* has the shortest lag period at 42°C, followed by 37°C, and 45°C. The same general pattern followed under aerobic conditions but the strain grew much slower and had much longer lag phase. The final cell densities obtained for *L. MONT4* grown at any growth temperature under anaerobic conditions are significantly higher than those obtained for *L. MONT4* grown at any growth temperature under aerobic conditions. The profiles of acid production, glucose utilization, and lactic acid production were tightly associated with growth. *L. MONT4* does not produce any ethanol at any growth temperature tested under either aerobic or anaerobic conditions. These results suggested that *L. MONT4* does not have an obvious alternative pyruvate-utilizing pathway.

We also started to clone the putative promoter of the *lldh* gene from *L. MONT4*. Previously, the *lldh* structural gene from *L. MONT4* was cloned in *E. coli* DH5a using degenerate primers designed based on sequenced *lldh* genes from lactic acid bacteria. The objective of this experiment is to clone the complete *lldh* gene from *L. MONT4*, including the gene expression signals. We believe that the putative constitutive *lldh* promoter is a strong promoter and can be placed in front of the *pdc adh* operon to enhance expression levels. The chromosomal DNA from *L. MONT4* was digested with a variety of restriction enzymes. Southern hybridizations were performed using the digested chromosomal DNA preparations and a 785-bp *lldh* probe. The chromosomal regions that hybridized to the *lldh* probe were determined. This information was used to map both upstream and downstream regions of the chromosomal *lldh* gene. Based on the newly constructed map of the *lldh* gene, several restriction enzymes were selected as the cloning enzymes. We have identified several positive clones and the DNA will be sequenced.

Select Second Generation Microorganisms for Ethanol Production From Lignocellulosic Biomass

Lactobacillus is capable of fermenting many of the sugars commonly found in biomass and offers potential advantages in biomass-to-ethanol fermentations including high ethanol tolerance, resistance to inhibitors present in hydrolysates, fermentation at low pH and thermotolerance. *Lactobacillus* was identified as a promising genus of microorganisms for metabolic engineering for ethanol production in our previous study. In particular, a unique *Lactobacillus* strain, Mont4, capable of growth up to 45°C, is a promising microorganism for metabolic engineering for ethanol production from renewable lignocellulosic biomass

because of its broad substrate fermentation range and its unique ability to metabolize pentose sugars such as L-arabinose and D-ribose through a homofermentative pathway.

Previous study has shown that *L. Mont4* can tolerate 80% (v/v) overlimed poplar wood hydrolysate and 8% (v/v) ethanol at 37 °C, respectively. To access the capability of the potential ethanologen, *L. Mont4*, we conducted a study using factorial design to evaluate the inhibition of cell-growth and glucose conversion by *L. Mont4* in the presence of overlimed hardwood hydrolysate and exogenous ethanol. Cell-growth and sugar-conversion by fermentation to lactic acid was determined by starting cultures at low ($A_{600}=0.25$) and at intermediate ($A_{600}=2.0$) cell densities. The pH was moderated in selected cultures for evaluating glucose conversion by adding finely powdered CaCO_3 .

Growth is more sensitive to all combinations of inhibitors than is glucose conversion. The effects are more pronounced at the higher temperatures. 5 % (w/v) ethanol appears to be more toxic than 80% (v/v) hydrolysate in cultures without pH moderation.

Moderating the culture pH above 5.0 significantly reduces inhibition for all conditions, especially at higher temperatures. pH moderation reduces inhibition of both hydrolysate and hydrolysate/ethanol mixtures, as expected. Interestingly, pH moderation also reduces inhibition by ethanol alone. All conversions were increased to 100 %, except at the highest concentrations of both hydrolysate and ethanol at the higher temperatures (80% for 41 °C and 30% for 45°C).

The above results demonstrated that *L. MONT4* can tolerate well in 5 % (w/v) ethanol and overlimed 80% (v/v) hydrolysate with moderating pH above 5 at 41 °C.

Developing Arabinose-Fermenting Yeast

This is a funds-in CRADA with CRA/NCGA. We previously reported that we cloned three arabinose-assimilating genes *araA*, *araB*, and *araD* in TRP1 vector and transformed these plasmids to an appropriate yeast strain, respectively. The transformants were isolated and assayed for the enzyme activities. The L-ribulokinase (*araB*) and L-ribulose-5-P-4-epimerase (*araD*) were expressed at high level and L-arabinose isomerase (*araA*) was expressed at low level. We have since constructed two sets of *araA*, *araB*, and *araD* expression plasmids in the HIS3 and URA3 yeast vectors. We also constructed a set of seven yeast strains that carry the expression plasmids for each of the *ara* genes with all possible combinations of the yeast selection markers (TRP1, HIS3, and URA3). In addition, we constructed the control strain that carries the three empty vectors. The transformed strains were assayed for the three enzyme activities. Growth tests with these strains revealed that none of them grew on L-arabinose. We have conducted cell-free fermentations with the strains carrying the three *ara* expression plasmids and the control transformants. The extracts from cells grown under the most favorable conditions convert some arabinose to ethanol after prolonged incubation whereas the control strain does not produce any ethanol under the same conditions. This result is very encourage. We are now further characterizing these strains for *in vivo* fermentation.

Scientific Publications, Presentations, and Other Activities

Patents or Record of Invention (ROI) Filed

M. Zhang and YC Chou filed two ROIs on “stable xylose and arabinose-fermenting *Zymomonas* strains” and “Method for gene inactivation in *Zymomonas*”

Project Operation

The SDT held two CRADA review meetings with the CRA/NCGA on January 7 and March 31, 2000. We provided the technical progress to the technical panel for both CRADAs (*Zymomonas* and yeast). Technical progress was presented to the representatives from Cargill, A.E. Staley, ADM, and CRA. We have met all the CRADA research objectives. The feedback received from CRA is very positive.

Publications, Posters, and Presentations at Scientific Meetings

An abstract entitled “Genetic improvement of *Zymomonas mobilis* for ethanol production: chromosomal integration of the xylose and arabinose-Fermenting genes by M. Zhang, Y.C. Chou, A. Mohagheghi, K. Evans, S. Milstrey, X.K. Lai, and M. Finkelstein was submitted for oral presentation at the 22nd Symposium on Biotechnology for Fuel and Chemicals in Gatlinburg, TN, May 6-11, 2000.

An abstract entitled “Cofermentation of glucose and xylose by integrated strains of *Zymomonas mobilis*” by K. Evans, A. Mohagheghi, Y.C. Chou, and M. Zhang was submitted for poster presentation at the 22nd Symposium on Biotechnology for Fuel and Chemicals in Gatlinburg, TN, May 6-11, 2000.

An abstract entitled “Evaluation of *Lactobacillus* MONT4 for cell-growth and glucose conversion in the presence of overlimed hardwood hydrolysate and ethnaol” by A. Mohagheghi, F. A. Keller, and M. Zhang was submitted for poster presentation at the 22nd Symposium on Biotechnology for Fuel and Chemicals in Gatlinburg, TN, May 6-11, 2000.

ENZYMATIC PROCESS DEVELOPMENT

Summary of Technical Achievements or Results

Corn stover process development activities remained the focus of the Enzymatic Process Development's (EPD's) work during the reporting period. The main emphasis was on improving our understanding of corn stover composition and developing and demonstrating rapid compositional analysis methods and the ability to continuously pretreat corn stover feedstock at the pilot scale. A secondary emphasis was to develop methodologies for closing the carbon balance on fungal-based cellulase enzyme production using insoluble cellulosic substrates. The EPD team achieved several important technical accomplishments related to these objectives during the reporting period:

- We successfully completed a C-milestone reporting on the first year of a multi-year study examining the composition and compositional stability of corn stover during long-term outdoor storage.
- We completed on schedule a C-milestone to develop and demonstrate the capability to continuously pretreat corn stover in the Alternative Fuels User Facility's (AFUF) pilot-scale Sunds Hydrolyzer pretreatment reactor.
- We exceeded a P-milestone to develop a near infrared spectroscopy-based (NIRS-based) method for rapidly determining the chemical composition of corn stover. A NIRS-based rapid analysis method for corn stover was successfully developed and applied to analyze corn stover samples.
- EPD researchers completed on-budget and ahead of schedule a C-milestone to demonstrate methodology for closing carbon mass balances on cellulase enzyme production using insoluble cellulosic substrates.

General Technical/Scientific Progress

EPD team activities remain focused on core technology development. Our objective continues to be to develop integrated enzymatic cellulose hydrolysis-based process technology that performs at levels consistent with the near-term commercialization objectives outlined in the Multi Year Technical Plan (MYTP). During the reporting period, we completed our transition from using yellow poplar sawdust as our primary biomass feedstock, and we are now focusing solely on corn stover feedstock (although pretreated yellow poplar continues to be used as a reference substrate where appropriate).

With the exception of the change in primary feedstock from yellow poplar to corn stover, the scope of our process development efforts remains similar to those described for the last reporting period. In particular, the enzymatic cellulose hydrolysis-based process that we are using as a base-line against which other process options are compared is based upon the following components:

- co-current dilute sulfuric acid prehydrolysis or pretreatment to solubilize the hemicellulosic sugars and increase the enzymatic digestibility of the cellulose;
- enzymatic cellulose conversion carried out simultaneously with mixed biomass sugar ethanol fermentation using a simultaneous saccharification and cofermentation (SSCF) process configuration;
- mixed biomass sugar fermentation using an adapted variant of xylose-fermenting *Zymomonas mobilis* as the SSCF microorganism;
- aerobic submerged cellulase enzyme production by the fungus *Trichoderma reesei* using cellulose as the primary carbon source.

The team's major efforts over the past 6 months focused on developing the know-how and capabilities to carry out effective corn stover bioconversion process development. Specific objectives were to characterize corn stover composition, develop rapid spectroscopy-based methods for its compositional analysis, and to demonstrate the ability to continuously pretreat corn stover at the pilot scale. An important but secondary thrust was to develop methodologies for closing the carbon balance on *T. reesei*-based cellulase enzyme production using insoluble cellulosic substrates.

Corn Stover Compositional Stability Study

In April, we successfully completed a C-milestone reporting on the first year (16 months) of a multi-year study examining the composition and compositional stability of corn stover during long-term outdoor storage (FY00 Milestone #7). This information is needed to understand the extent to which long-term storage influences composition and thereby process economics; corn stover will lose value as a feedstock if carbohydrates are slowly degraded by microbial action.

Three rounds of sampling were completed on large round bales of corn stover stored at Biomass Agri-Product's (B/MAP) (formerly Great Lakes Chemical) Harlan, Iowa, collection facility. Compositional analyses using wet chemical methods were performed on a subset of the samples. Results show that the average compositions (and ± 1 standard deviations) for the stored Harlan corn stover components are as follows (dry weight basis): 33.3% ($\pm 2.8\%$) glucan, 19.5% ($\pm 2.8\%$) xylan, 18.9% ($\pm 3.7\%$) insoluble lignin, 2.2% ($\pm 0.3\%$) acid soluble lignin, 2.9% ($\pm 0.5\%$) arabinan, 1.8% ($\pm 0.4\%$) galactan, 0.6% ($\pm 0.2\%$) mannan, 1.9% ($\pm 0.8\%$) acetyl, 5.0% ($\pm 1.9\%$) ethanol soluble extractives, and 8.5% ($\pm 5.6\%$) ash. Improved bale sampling and sample handling techniques were implemented during the course of this work, significantly reducing the high levels of compositional variability caused by contaminating dirt (indicated by high ash levels) in the samples. The higher standard deviations on the individual component levels provide a good example of the large natural compositional variability that we believe exists in corn stover. With the exception of particularly high average levels of ash and somewhat lower average levels of glucan, these compositional results are similar to those previously reported in the literature. It is expected that the lower glucan values are partially due to the high levels of dirt present in the sampled corn stover bales; glucan levels are higher in washed samples. The relatively large

compositional variability suggests that reporting compositional ranges may be more meaningful for economic assessments than reporting compositional averages.

In terms of compositional stability, the first year study results demonstrate that small but statistically significant changes in corn stover composition occur during long-term storage. Additional measurements of bale weight losses over time will need to be obtained to interpret the stability trend data. While these results provide a solid foundation for improving our understanding of corn stover composition and stability, this study will need to be extended for several more years. It will also need to be expanded to include corn stover produced in other regions of the United States (especially within the corn belt) to understand the true compositional variation in corn stover and its technoeconomic significance. Preliminary economic sensitivity analyses show that compositional variability of the magnitude observed in the first year of this study has substantial impact on bioethanol process economics. In particular, a 1% change in feedstock carbohydrate composition is predicted to increase the minimum ethanol selling price by approximately 2.5¢ per gallon. The fact that even relatively small changes in feedstock composition can significantly affect production cost underscores the need to have more accurate information about feedstock composition. It is also important to recognize that herbaceous biomass species and agricultural residues like corn stover contain lower levels of carbohydrates than short rotation hardwoods like poplars. Therefore the advantage of using corn stover feedstock to expand domestic fuel ethanol production lies less in its carbohydrate composition than in its abundance and widespread availability.

Development and Application of Near Infrared Spectroscopy-Based Rapid Analysis Methods

As discussed in previous reports, rapid spectroscopy-based methods for determining the composition of biomass feedstock like corn stover promise to greatly reduce the process development bottleneck caused by the significant time and cost of biomass compositional analysis using traditional wet chemical methods. Recognizing the benefits to improved research efficiency, the Ethanol Project has continued to support development and application of rapid analysis methods.

In September, the EPD team successfully completed and exceeded a P-milestone to develop a NIRS-based method for rapidly determining the chemical composition of corn stover. This NIRS-based rapid analysis method was then successfully applied to analyze corn stover samples. The milestone objectives were exceeded in two respects. First, the team developed a complimentary NIRS-based rapid analysis method for determining moisture levels in corn stover samples while minimizing sample preparation. This secondary moisture analysis method provides an efficient means of screening samples to ensure that the NIRS-based compositional analysis method can be accurately applied. Second, these rapid analysis methods were tested extensively by analyzing approximately 650 corn stover feedstock and storage study samples. The application of these new methods allowed many more samples to be analyzed than would have been economically feasible using expensive and time consuming traditional wet chemical analysis techniques. We estimate that using traditional methods to analyze these 650 corn stover samples would have taken about 15 months and cost roughly \$700,000. In contrast, applying the rapid analysis methods took about 10 weeks and cost under \$10,000 (neglecting the cost

to develop the method). These results show the powerful benefits to research productivity that can accrue once rapid analysis methods are developed and validated.

In FY01, we plan to expand the existing corn stover composition calibration sample set to further increase the accuracy and robustness of the NIRS-based corn stover compositional analysis method. Also, a similar NIRS-based rapid analysis method will be developed for determining the composition of pretreated corn stover solids.

Corn Stover Pretreatment

The EPD team completed on schedule a C-milestone to develop and demonstrate the capability to continuously pretreat corn stover in the AFUF's pilot-scale Sunds Hydrolyzer pretreatment reactor (FY00 Milestone #27). The original milestone goal had been to perform a response surface analysis of corn stover pretreatment and to identify operating conditions that would enable monomeric xylose yields of 75% of theoretical to be achieved. This objective was scaled back to demonstrating the ability to pretreat corn stover after unforeseen problems arose with our ability to effectively handle the extremely heterogeneous and low bulk density corn stover feedstock in the PDU's feed handling and Sunds reactor pretreatment systems. Significant modifications and repairs were made to the feed handling and pretreatment equipment in order to develop the capability to reliably process corn stover through this system.

First, the continuous pilot scale Sunds Hydrolyzer pretreatment reactor system was reassembled and tested for operability using corn stover. Significant repairs and modifications to the feed handling and pretreatment reactor system were completed before this testing could occur. Critical repairs made to the system included installing a new screen and additional parts (i.e., rebuilding) in the plug flow feeder as well as installing new and repaired parts for the vertical impregnator section of the Sunds reactor itself. In addition, adjustments were made to the screw auger gear motor box that controls the rate at which feedstock material is fed from the feed hopper. Initial shakedown testing of the rebuilt Sunds hydrolyzer reactor system using corn stover (after a water wash and air drying) showed that the refurbished Sunds reactor system could handle this material reasonably well. The pug mill, cross feeder, and plug feeder all worked well enough to achieve steady feeding of the Sunds reactor. Some minor issues were identified, however, that need to be acted on. First, a small problem was identified with the squeeze pump. This problem was overcome by using a different pump that we had on site. Second, minor additional modifications were made to the feed hopper and belt feeder system to improve their operability. In conjunction with these activities, we procured a large supply (approximately 10 tons) of corn stover from B/MAP in Harlan, Iowa.

Despite having made numerous and substantial modifications to the PDU's front end biomass handling systems, mechanical and operational difficulties continued to arise compromising our ability to continuously feed corn stover into the Sunds pretreatment reactor at a steady target rate. According to T. Schechinger of B/MAP, a company with significant experience handling corn stover (see more on this below), highly problematic feedstock "roping" occurs when the longest length of the thinnest corn stover fibers exceeds about 3 inches. The longest fiber length in the coarsely milled corn stover feedstock supplied to date

to NREL by B/MAP is approximately 6 inches. However, significant size reduction takes place in the wash tank (hydropulper) used to remove dirt from the material and roughly 90% of the washed material is less than 2 inches in length. While severe roping was not observed in the PDU feed hopper, the feed auger was underpowered at the low screw speeds needed to process the material. As a result, the hopper had to be run almost empty to be able to continuously feed material. While this greatly compromised our ability to run for long periods of time, continuing shakedown work with the system occurred using this work-around approach.

Eventually, it was determined that a more powerful motor with higher torque was needed to drive the feed hopper auger and achieve reasonably steady state operation. A larger feed screw shaft and higher torque drive were procured and installed. Testing showed that these modifications enabled the feed hopper to handle corn stover much more effectively. In particular, the feed hopper could be operated nearly fully loaded and the stability in the rate of feedstock fed to the pretreatment system was substantially improved. The new motor drive stalled, however, when it was operated at low speeds (less than 10% of the maximum output of the variable frequency drive), so replacement of the variable frequency drive with a newer unit is planned to cost-effectively overcome this problem. Improved variable frequency drives are now available that do not suffer from this limitation.

A formal request was made to reduce the expectations for EPD's September pretreatment C-milestone, since the unanticipated amount of work required to get the Sunds Hydrolyzer pretreatment and feeding systems operational for coarsely milled corn stover feedstock had prevented a detailed pretreatment response surface from being generated. The reduced milestone goal was to demonstrate pretreatment of corn stover in the pilot scale Sunds Hydrolyzer pretreatment reactor system under one or more different conditions. This reduced milestone expectation would not enable corn stover pretreatment performance to be characterized as a function of operating conditions but would produce material that would enable a preliminary assessment of pretreatment system operability.

Ultimately, a single pretreatment condition was tested (using a reaction temperature of 165°C, residence time of 8 minutes, sulfuric acid concentration of 1.5%, and solids loading of approximately 23%). A monomeric xylose yield of 60.7% was achieved under these conditions, which is comparable to the performance obtained in an earlier small-scale steam gun pretreatment carried out under similar conditions. Most importantly, the feed handling and pretreatment systems performed well during this pretreatment test, demonstrating that the substantially modified system is now ready to be used to continuously pretreat corn stover. In FY01, this system will be used to carry out a more detailed response surface exploration of corn stover pretreatment performance as a function of feedstock characteristics and pretreatment operating conditions.

Other Corn Stover-Related Activities

EPD researchers visited with representatives of B/MAP in August to meet with their consultant T. Schechinger and president L. McLaughlin. The purpose of the meeting was to: 1) tour B/MAP's Harlan IA corn stover collection, storage, and processing facility; 2) discuss issues related to corn stover compositional variability and assess what B/MAP could do to provide the Ethanol Project with various grades of pre-processed corn stover for pretreatment

and bioconversion testing; and 3) to explore B/MAP's vision of what would be involved in developing an infrastructure to cost-effectively supply corn stover to a large-scale bioethanol production and bio-based products industry. The trip was very informative and succeeded in meeting all of its objectives. Highlights include identifying previously unrecognized issues beyond geographical and climatic year-to-year variability that may be contributing to the relatively low cellulose levels observed in the corn stover samples that we've collected from the Harlan site relative to those reported in the literature. First, cultivation densities (i.e., the number of plants per acre) have increased significantly and cultivated varieties have changed over the past decade as grain yields have increased, perhaps to the detriment of cellulose levels in the stover portion. Second, cellulose levels are likely to be influenced by the time at which the plant is harvested. Higher levels of carbohydrates are expected to occur in the stover portion of the corn plant earlier in the season and to decrease with time as plant carbohydrate is diverted to the corn kernels. To the extent resources permit, further work will be carried out to test these hypotheses. The B/MAP representatives also indicated that they could supply the Ethanol Project with several different grades of washed, milled, and dried corn stover for pretreatment and bioconversion testing. For preliminary budgeting purposes, they estimated that approximately \$10,000 would cover the cost of running the Harlan plant for one full day, which would produce about 10 tons of material. They also believed that they would be able to provide the Ethanol Project with preliminary data within the next 6 months that could support baseline cost estimates for reduced collection costs and infrastructure requirement. T. Schechinger described many of his ideas for innovative corn stover collection, transportation, and storage methods that appear to offer the potential to significantly reduce the cost of delivering corn stover to large-scale bioprocessing facilities.

The EPD also began collaborating with the U.S. Department of Agriculture (USDA) researchers during the reporting period to explore the potential of obtaining higher value co-products from corn stover bioconversion process residues. Kevin Hicks, a plant science and technology research leader at the USDA Agricultural Research Service's Eastern Regional Research Center, in Wyndmoor, Pennsylvania, is interested in this collaboration because his group has identified extractable minor lipids in corn stover that survive pretreatment conditions and may command a premium market value. We recognize that identifying valuable co-products in corn stover-based bioethanol process residues facilitates the deployment of corn stover-based bioethanol production technologies and support this collaboration. We will provide the ARS researchers with sub-samples of compositionally characterized feedstock, process intermediates, and process residues as they become available during the course of our on-going process development activities.

Carbon Balancing of Insoluble Substrate-Based Cellulase Enzyme Production

In August, EPD researchers completed on-budget and ahead of schedule a milestone to demonstrate methodology for closing carbon mass balances on cellulase enzyme production using insoluble cellulosic substrates (FY00 C-milestone #15). While closing carbon mass balances is necessary to verify the quality of cellulase production process performance data and is essential to confirm process yields, this effort is pioneering because no previous equally rigorous carbon balances on insoluble substrate-based cellulase enzyme production have been reported. It should also be noted that a large portion of this work was carried out by J.C. Sáez, a chemical engineering masters student

at the University of Puerto Rico, Mayaguez. J.C. Sáez interned with the EPD team from June 1999 to July 2000 and was a participant in DOE's OFD-sponsored Biofuels Research Initiative for Student Advancement (BRISA) program.

The highlights of this work consisted of performing 13 separate cellulase production experiments in 7-L fermentors using *T. reesei* grown in three different substrates: 9 runs were performed using a purified cellulose preparation (Solka-floc), 3 runs using soluble glucose, and 1 run using soluble lactose. In all cases, all the carbon flows in all carbon-containing input and output process streams (i.e., gaseous, liquid, and solid) were quantified using appropriate analytical techniques developed during the course of the work to quantify the amount of carbon in each stream. Cellulose and fungal cell mass levels were determined using total dry weight measurements in combination with a near infra-red spectroscopic method reported on in a March 2000 P-milestone report. Levels of carbon in the fungal cell mass were determined using elemental analysis. Carbon dioxide production was quantified using a gas mass spectrometer and a gas mass flow meter. Uncertainty analysis was used to calculate 95% confidence limits on carbon mass balance methodology to assess its accuracy.

Carbon mass balance closures using Solka-floc substrate were between 90% and 100% during the first 48 h of cultivation but increased to approximately 101%-135% from 72 h to the end of the cultivation at 168 h. Carbon mass balance closures for soluble sugar substrates range from 92% to 127% over the entire course of the cultivation. Uncertainty analysis showed that the 95% confidence intervals for carbon mass balance closures were approximately plus or minus 12% for results obtained after 48 h of cultivation. Thus, many of the carbon mass balance results obtained in the later stages of cultivation do not bracket 100% closure within the 95% confidence interval, which indicates that measurement problems exist with quantifying carbon flow late in the process. Results of the uncertainty analysis suggest that further improvement in carbon mass balance closures for cellulase production are achievable, particularly during the latter stages of the cultivation. Close examination of the data points to measurement of carbon dioxide production as the probable cause of the high carbon mass balance closures late in the cultivation. Both factors associated with quantifying carbon dioxide production – measuring gas composition and gas mass flow rate – are suspect, but additional work is required to resolve the measurement problems. On a positive note, the carbon mass balances are reasonably good at the early stages of cultivation (24 h to 48 h), indicating that the underlying carbon balance methodology and most of the analytical methods are sound.

Other Outreach Activities

We sent out a letter and supporting documents to G. Luli at BC International (BCI) describing EPD's planned future work activities in the area of cellulase enzyme process development and solicited feedback on these plans. Initial comments from BCI indicate that they support the plan. Follow up information and citations on enzyme production technology were being provided to BCI to facilitate their in-house cellulase production work. D. Schell and J. McMillan subsequently met with BCI scientists G. Luli (Principal Investigator), B. Wood, and J. Lee at BCI's research facility located at the University of Florida's Sid Martin Biotechnology Development Institute in Alachua, Florida, to review technical progress and discuss future research directions for BCI's subcontract (XCO-0-

29107-01). The objective of this subcontract is for BCI to assess the readiness of enzymatic cellulose hydrolysis-based bioethanol production from pretreated bagasse and pretreated rice straw using currently available cellulase production technology and BCI's fermentation strains. Several quality control issues were identified and specific recommendations were made to improve data quality and interpretability. Future work focuses on completing enzymatic saccharification and simultaneous saccharification and fermentation (SSF) evaluations of commercial cellulase preparations using pretreated bagasse and pretreated rice straw and performing cellulase production runs using these substrates. A 3-month no-cost extension was put in place to permit BCI to finish their planned work.

In September, Juan Carlos Sáez, a chemical engineering masters of science student at the University of Puerto Rico, Mayaguez (UPR, Mayaguez), successfully defended his masters thesis on carbon mass balancing of *T. reesei*-based cellulase enzyme production using insoluble substrates to his thesis committee, which was comprised of Professors N. Cardona (Biology), F. Padron, J. Colucci, and L. Salicetti (all from Chemical Engineering), and J. McMillan from NREL, where his thesis research was carried out. Mr. Sáez is the first graduate student from UPR, Mayaguez, to complete his thesis research at NREL under the DOE's OFD-sponsored Biofuels BRISA program.

Scientific Publications, Presentations, and Other Activities

Publications, Posters, and Presentations and Scientific Meetings

The following papers and posters were presented at the 22nd Symposium on Biotechnology for Fuels and Chemicals held May 7-11 in Gatlinburg, Tennessee:

"Bioethanol Production Using Simultaneous Saccharification and Cofermentation: Impact of Cellulase Enzyme Quality," by J.D. McMillan (speaker), N. Dowe, J.D. Farmer, J. Hamilton, R. Lyons, A. Mohagheghi, M.M. Newman, J.C. Sáez, D.J. Schell, D.W. Templeton, and A. Tholudur. Oral presentation 6-05 (substitute for withdrawn paper).

"Influence of Operating Conditions and Vessel Size on Oxygen Transfer during Cellulase Production," D. Schell (presenter), J. Farmer, J. Hamilton, B. Lyons, J.C. Sáez and A. Tholudur. Poster presentation 6-19.

"Integrated Simultaneous Saccharification and Cofermentation Using Whole-cellulase Enzyme and Xylose-fermenting *Zymomonas mobilis*," by N. Dowe (presenter), M.M. Newman, A. Mohagheghi, and J.D. McMillan. Poster presentation 6-20.

The following papers and chapters were published or submitted during the reporting period:

Hayward, T.K.; Hamilton, J.; Tholudur, A.; McMillan, J.D. (2000). "Improvements in Titer, Productivity and Yield using Solka-floc for Cellulase Production." *Appl. Biochem. Biotechnol.* 84-86: pp. 859-874.

Lawford, H.G.; Rousseau, J.D.; Mohagheghi, A.; McMillan, J.D. (2000). "Continuous Fermentation Studies with Xylose-Utilizing Recombinant *Zymomonas mobilis*." *Appl. Biochem. Biotechnol.* 84-86: pp. 295-310.

Ranatunga, T.D.; Jervis, J.; Helm, R.F.; McMillan, J.D.; Wooley, R.J. (2000). "Overliming of Dilute Acid Pretreated Lignocellulosics: The fate of inorganics, uronic acids and ether-soluble organics." *Enzyme and Microbial Technology*, 27: pp. 240-247.

Huang, Z.; Maness, P-C.; Dowe, N.; Mohagheghi, A.; Newman, M.; McMillan, J.D. (2000). "Rapid Detection of *Zymomonas mobilis* Redox Activity Using 5-cyano-2,3-tolyl-tetrazolium chloride (CTC)." *Biotechniques*, 29(3): pp. 424-428.

McMillan, J.D.; Dowe, N.; Mohagheghi, A.; Newman, M.M. (2000). "Assessing the Efficacy of Cellulase Enzyme Preparations under Simultaneous Saccharification and Fermentation Processing Conditions," Chapter 9 in Glycosyl Hydrolases for Biomass Conversion, M. Himmel, ed. *ACS Symposium Series 769*, American Chemical Society: Washington, DC, pp. 144-167.

Tholudur, A.; Hames, B.; Meglen, R.; Hayward, T.; Saez, J.; Hamilton, J.; McMillan, J. (2000). "A Near Infrared Spectroscopy-based Analysis Technique for Quantifying Cell Mass in the Presence of Insoluble Solids." *Biotechnology Progress*. Submitted.

LIGNIN CONVERSION TO FUELS

Summary of Technical Achievements or Results

The goal of the Lignin Conversion to Fuels task is to develop processes for converting lignin into valuable fuel blending agents. Lignin is the biomass component with the highest energy content (9000 - 11000 Btu/lb vs. 7300 - 7500 for cellulose), and can be burned to provide process heat. The novel processes developed in this task are expected to increase the value of the lignin relative to its fuel value, thereby improving the overall economics of biomass to liquid fuels conversion technologies.

Given the molecular nature of lignin it is conceivable to convert lignin into high-octane fuel additives. However, we must ensure that the lignin-derived products are compatible with the evolving regulations for transportation fuels. From lignin it is possible to make aromatic hydrocarbons and/or ethers that fit within the specifications for reformulated gasoline. This project is thus targeted to obtain the necessary scientific and technological data with which to evaluate the economics and markets for converting ethanol process lignins into valuable fuel additives.

The main source of lignin from Biomass-to-Ethanol processes is the high molecular weight lignin present in the solid residue remaining after dilute acid pretreatment followed by SSF. The lignin is soluble in basic solutions, and can be readily separated from the protein, unconverted carbohydrate, and other non-lignin materials present in the residue by solubilization. NREL, the University of Utah (UU), and Sandia National Laboratory (SNL) are developing process strategies to depolymerize the lignin by base catalysis. To minimize the cost of base recovery only low levels of soluble base will be used. Solid base catalysts are being developed to produce higher levels of depolymerization. The resulting intermediate is a base catalyzed depolymerized (BCD) product that consists of monomeric and oligomeric phenolics.

The BCD product is isolated by solvent extraction and converted by one of the following catalytic upgrading routes. Hydroprocessing (HPR) utilizes catalysts with hydrodeoxygenation (HDO) and hydrocracking (HCR) activities to remove all remaining oxygen and break the interaromatic C-C linkages to produce a mixture of monomeric aromatic hydrocarbons very similar to components already found in gasoline. Alternatively, mild hydrotreatment (HT) plus etherification can be used to yield aromatic ethers, oxygenates, that could be used as octane enhancers. HT cracks C-C linkages with only partial deoxygenation. The HT product is a mixture of phenols that can be etherified. Currently, the main objective of the task is to optimize the yield of the HPR process to make a hydrocarbon product in the gasoline boiling range.

UU has scaled up the BCD process from bench scale autoclaves to a flow reactor capable of processing at least 100 g/h of lignin. A flow reactor for the HPR step has also been constructed and is being used to generate larger samples of the lignin-derived fuel additive for fuel property testing.

General Technical/Scientific Progress

A significant milestone has been achieved in the last 6 months with the delivery of the first large sample (more than 150 g) of the lignin-derived hydrocarbon product by Professor Shabtai at the UU. The product was made from lignin depolymerized by base catalysis in a flow reactor system. The depolymerized lignin was then hydroprocessed in a stirred autoclave in a single stage using a mixture of hydrodeoxygenation and hydrocracking catalysts. A sufficient amount of the hydrocarbon product was obtained by accumulating the product from several runs in the autoclave. Yields of 76% and 58% (on a weight basis) were reported by Professor Shabtai for the depolymerization and hydroprocessing stages, respectively. Overall this is a yield of 44% for conversion of lignin into hydrocarbon product on a weight basis, which is equivalent to 60% of the theoretical maximum. Professor Shabtai expects yields to improve by further optimizing the BCD and HPR reactions and by transferring the HPR reaction to a flow reactor system.

The lignin-derived hydrocarbon product has undergone extensive chemical analysis at NREL and was sent for octane testing to a fuel-testing laboratory in Houston, TX. For comparison, a sample of a racing fuel (octane 101) was sent for testing with the lignin-derived fuel. Both samples were tested as 10% blends in unoxygenated regular gasoline (octane 87.0). The two blended samples both gave slightly higher octanes than the base fuel with the blend containing the lignin-derived fuel measuring slightly higher (88.0) than the blend containing the racing fuel (87.5). The reproducibility of these numbers was quoted to be about 0.5 of an octane number. Although the blending octane number of the lignin-derived product based on this measurement (97) is somewhat lower than we would eventually like to achieve (octane 110), this is a good result for the first measurement of octane on a lignin-derived hydrocarbon gasoline additive. When larger samples become available higher blend levels (50 – 100%) will be tested so that blending octane values can be determined with greater confidence.

The volatility of the large sample of lignin-derived hydrocarbon product was measured by simulated distillation. The results indicate that the sample contained a significant amount of material (about 35%) that was above the normal gasoline boiling range (above 200 °C), and even some material (about 8%) that could have a boiling point above 250 °C. The results obtained by simulated distillation need to be corroborated by the standard ASTM distillation method, but this requires at least 100 g of the product. This will be done as soon as a larger sample of the product can be obtained. Chemical analysis of the product has shown that hydrodeoxygenation was not complete when this sample was produced. The sample appears to contain 25-35% of phenols. Generation of previous hydrocarbon products did not have this problem and so it is not anticipated that there will be any problem in obtaining completely deoxygenated products in the future. While the properties of this initial product do not meet our ideal specifications, we believe that we will be able to produce larger samples of lignin-derived hydrocarbon products that can be used for fuel property testing and show that a commercially interesting hydrocarbon fuel can be made from Ethanol Process lignins.

Recent studies performed at SNL on the use of mixed solid and soluble bases in the lignin depolymerization reaction have found that this potentially cheaper base system is effective in the lignin depolymerization reaction. Currently studies at SNL are aimed at gaining an

understanding of the effects of mixing and lignin solubility on the yields of depolymerized product.

A report entitled "Review Mechanism for Registering an Octane Enhancer," by J.E. Sinor Consultants was recently completed. J.E. Sinor is a subcontractor to NREL who has significant experience in assessing the fuels market. The objective of this review was to determine what steps would be necessary to get lignin-derived products approved for sale.

Gasoline and gasoline additives are regulated at the federal level by the U.S. Environmental Protection Agency (EPA) under Section 211 of the Clean Air Act. At the state level, only California has a federally authorized comprehensive regulatory program for gasoline, which is carried out by the California Air Resources Board.

Key conclusions from Dr. Sinor's review include:

- Completing all of the testing required to register a new additive may cost approximately \$3 million and require 2 to 3 years to complete.
- An aromatic ether is unlikely to be granted a waiver of testing requirements under the "substantially similar" rule.
- Because of the incidents of ground water contamination by MTBE, it has already been banned in California and is likely to be completely phased out.
- The ban on MTBE could have a carry-over effect of banning all ethers.
- The above strongly suggests that aromatic ethers will not be a practical product, at least in the near term.
- It is possible that an alkyl benzene additive of sufficient purity, consisting of only carbon and hydrogen, would not require registration.
- If the alkyl benzene additive contains impurities consisting only of oxygen, nitrogen and/or sulfur, it might be possible to register it under the "substantially similar" guidelines to avoid lengthy and expensive testing requirements.
- No matter which pathway is followed, the EPA reserves the right to require additional health effects testing.

Consequently, the aromatic hydrocarbon product must remain the primary product of our research. The cost and effort required to gain acceptance for an aromatic ether product is likely to be prohibitive even if its chemical properties, which are significantly different from MTBE, are favorable for its use as a gasoline additive. The hydrocarbon product should have the same composition as a fraction of the gasoline in use today and so is unlikely to face any significant regulatory hurdles to its use as a gasoline additive.

Scientific Publications, Presentations, and Other Activities

A poster entitled “Conversion of Lignin into High Octane Fuel Additives,” by E. Chornet, D.K. Johnson, W. Zmierczak, and J. Shabtai, was presented at the Conference on Progress in Thermochemical Biomass Conversion in Tyrol, Austria, September 17-22, 2000.

The “Review of Market for Octane Enhancers,” prepared by J.E. Sinor Consultants Inc., has been published as an NREL report (NREL/SR-580-28193).

COLLABORATIVE INDUSTRIAL PROCESS DEVELOPMENT

Summary of Technical Achievements or Results

(The following contains NREL protected information regarding the effect of pressing of biomass feedstock on sugar yield; RI#00-52)

A revised conceptual process design of the NREL two-stage dilute acid hydrolysis technology was provided to Merrick and Company (Aurora, CO) for their consideration in an evaluation of currently available biomass ethanol technologies for SEAlaska Corp. Merrick has issued a drafted report indicating that NREL technology is the leading contender.

A P-milestone entitled "Preliminary Results of Dilute Acid Pretreatment of Corn Stover Using the 4-L Steam Digester," was completed. A series of pretreatment experiments were carried out at 160°-180°C, 1% sulfuric acid, and 3-14 min. The lower temperatures and long pretreatment times were selected so that the data can be used for scale-up experiments using the continuous Sunds Hydrolyzer. A maximum total soluble xylose (monomeric and oligomeric) yield of 77% was achieved at 180°C and 3-min residence time. The enzymatic cellulose digestibility of the pretreated solids was 90-95% of theoretical when using an enzyme loading of 26 FPU/g cellulose in SSF assays. Based on these results, a second series of pretreatment experiments were performed at 190°C and 70-110 seconds (s). A maximum total soluble xylose yield of 93% was obtained at the 90-s pretreatment time. These high xylose recovery yields and cellulose digestibility exceed the stretch performance targets of 85% xylose yield and >80% cellulose digestibility.

A P-milestone entitled "Effect of Pressing Acid-impregnated Biomass Feedstock on Soluble Sugar Yields in Dilute-acid Pretreatment of Softwood and Corn Stover," was completed. This study compares sugar yields of pressed and non-pressed (air-dried) biomass at similar pretreatment conditions (i.e., essentially the same temperature, pretreatment time, acid concentration, and moisture content). In parallel experiments involving first-stage pretreatment of softwood forest thinnings, the mannose yields of pressed acid-impregnated wood chips were 71-73% compared to 79-89% for air-dried chips. The solids content of both feedstock were about 42%. Pressing feedstock has an even larger negative impact on glucose yield from second-stage dilute acid hydrolysis of softwood. In experiments using the same water-washed 1st-stage pretreated fibers, the glucose yield obtained from pressed material was 34% compared to 47% for the air-dried material. Preliminary experiments indicate that pressing also has a negative effect on xylose yield from pretreated corn stover.

We postulate that pressing causes the structure of the lignocellulosic matrix to collapse, resulting in poor heat and mass transfer. Non-uniform acid distribution and poor heat transfer cause "undercooking" of the center of the biomass particles. Steam cannot penetrate the collapsed pores and rapidly heat the biomass particles. Instead, heat is transferred to the surface as the steam condenses. Because densified biomass is a poor heat conductor, heat transfer from the surface to the interior of the biomass particle is

mainly via slow conduction. With most of the acid squeezed out of the interstitial spaces, little hydrolysis occurs even if the particles are at higher temperatures.

The negative impact of pressing biomass on sugar yield suggests that continuous biomass-hydrolyzer feeders should be carefully designed to minimize densification of biomass particles. Taper screw-feeders can compress the feedstock to as high as 70% solids content in order to seal the reactors against high internal pressure. Such severe densification of biomass particles would likely lead to low hydrolysis sugar yields.

A P-milestone entitled "Preliminary Results of Continuous Countercurrent Extraction of Hemicellulose from Pretreated Wood Residues," was completed. Based on experimental data collected from a 4-inch x 9-ft prototype counter-current screw extractor, an empirical equation predicts that soluble sugar above 95% can be achieved using a liquid-to-insoluble solid (L/IS) ratio of 3.0 for an 11-ft long extractor. This represents a significant reduction in water usage in comparison with drum washers, which typically require a L/IS ratio of about 4.0.

Two samples of lignin residues from softwood forest thinnings were produced for BCI for combustion characterization. The samples were insoluble solids obtained after ethanol fermentation and washing of the second-stage dilute acid hydrolysate.

General Technical/Scientific Progress

Two-stage Dilute Acid Hydrolysis Process Development

A revised conceptual process design of the NREL two-stage dilute acid hydrolysis technology was provided to Merrick and Company (Aurora, CO) for their consideration in evaluating currently available biomass ethanol technologies for SEAlaska Corp. Merrick has issued a draft report, which indicates that NREL technology is the leading contender. The NREL technology package contains several potentially patentable concepts.

Experimental results and process design data related to the NREL two-stage dilute acid hydrolysis technology are being compiled for evaluation by Bateman Engineering Inc. (Denver, CO). The purpose of this evaluation, as part of a Gate-3 review, is to examine the process design and recommend any further research and development work necessary for transferring this technology to industry for demonstration and commercialization.

Pretreatment of Corn Stover Using The 4-L Steam Gun

We have achieved 93% total soluble xylose yield (monomers and oligomers), which exceeds the stretch objective of 85%. A series of five pretreatment runs were made at 190°C, 1.06% sulfuric acid in the liquid of input material, and 70-110 sec. We believe the following factors contributed to the high xylose yield.

1. High temperature and short residence time. Literature information and previous steam gun pretreatment results (from corn stover and softwood feedstock) led us to choose the conditions for these runs.

2. We cut up the large, chunky particles. This "over-sized" fraction represents about 10% of the total feedstock.

Possible minor contributing factors:

1. Instead of being pressed, the acid-impregnated stover was air-dried to about 43% solids. We have seen that pressing to remove excess acid solution from the biomass has been detrimental to the sugar yields from softwoods. This problem appears to be less pronounced for corn stover.
2. We analyzed the liquor samples soon (within 2 days) after pretreatment. The sugars (especially the oligomers) seem to slowly disappear with storage in the refrigerator or freezer. We suspect that some of the sugars precipitate out with the lignin. Additional experiments are needed to confirm this limited observation (i.e., change in liquor composition) by high performance liquid chromatograph (HPLC) and Fourier transform infrared (FTIR).

Extraction of Soluble Sugars from Pretreated Wood

Extraction of soluble sugars from pretreated biomass is a required unit operation for two processes under development at NREL. For the two-stage dilute acid hydrolysis process, the soluble sugars from the first-stage hydrolysate slurry are recovered before the washed fibers are hydrolyzed in the second stage. For the enzymatic cellulose conversion process, the liquor is separated from the insoluble solids and then conditioned to improve the fermentability of the liquor.

A series of extraction experiments using a prototype 4-inch diameter x 9-ft long continuous countercurrent screw for the recovery of hemicellulose from pretreated softwood forest thinnings were completed. Soluble recoveries of 98%, 91%, and 77% were obtained with L/IS ratios of 5.6, 3.4, and 2.1, respectively. An empirical equation was developed to predict the performance of the screw extractor for first-stage pretreated softwood forest thinnings. The equation predicts that soluble sugar recovery above 95% can be obtained with an L/IS ratio as low as 3.0 for an 11-ft long screw. This represents a significant improvement over the drum washers, which typically require a L/IS ratio of about 4.0.

Bench-scale drainage tests were performed on first-stage and second-stage hydrolysates of softwood forest thinnings, pretreated yellow poplar, sawdust (by NREL), and pretreated yellow poplar chips (by TVA). The second-stage softwood hydrolysate has an extremely poor drainage rate. The pretreated yellow poplar materials also have a poor drainage rate of about one-third of the 1st-stage softwood drainage rate. The performance of the screw extractor was investigated using both pretreated yellow poplar materials. In the extraction runs with the pretreated yellow poplar materials, fine materials easily clogged the filtration system of the extractor. Also, the accumulation of fines at the bottom of the extractor slowed down the liquid extract rate significantly. This resulted in a dilute slurry in the upper part of the extractor, and the screw was not able to convey the solids to the discharge chute. Therefore, the countercurrent screw extractor, as configured, was found to be

unsuitable for processing biomass materials that have poor drainage rates such as the Sunds pretreated yellow poplar.

Scientific Publications, Presentations, and Other Activities

Scientific Meetings

K. Kim, M. Tucker, and Q. Nguyen presented three posters at the Twenty-Second Symposium on Biotechnology for Fuels and Chemicals. Papers for two of the posters (1 and 2) were submitted and subsequently accepted for publications in the Symposium Proceedings.

1. "Continuous Countercurrent Extraction of Hemicellulose from Pretreated Wood Residues," K.H. Kim, M.P. Tucker, F.A. Keller, A. Aden, and Q.A. Nguyen.
2. "Fourier Transform Infrared Quantitative Analysis of Sugars and Lignin in Pretreated Softwood Solid Residue," M.P. Tucker, Q.A. Nguyen, F.P. Eddy, K. Kadam, L.M. Gedvilas, and J.D. Webb.
3. "High-Yield Fermentation of Softwood Hydrolysate by Adapted Yeast followed by HPLC and FTIR Sample Analysis," F.A. Keller, M.P. Tucker, F.P. Eddy, and Q.A. Nguyen.

Scientific Journals

1. M.P. Tucker, R.K. Mitri, F.P. Eddy, Q.A. Nguyen, L.M. Gedvilas, and J.D. Webb (2000), "Fourier Transform Infrared Quantification of Sugars in Pretreated Biomass Liquors," *Applied Biochemistry and Biotechnology*, **84-86**, 39-50.
2. Q.A. Nguyen, M.P. Tucker, F.A. Keller, and F.P. Eddy (2000), "Two-stage Dilute-acid Pretreatment of Softwoods," *Applied Biochemistry and Biotechnology*, **84-86**, 561-576.

Patents or ROIs Filed

A patent application entitled "Dilute Acid/Metal Salt Hydrolysis of Lignocellulosic Biomass," was filed with the United States Patent and Trademark Office. The inventors are Q. Nguyen and M. Tucker.

The NREL Patent Management Committee recommended that MRI elect title to invention RI # 00-49 " Strain of Pichia Stipitis Yeast Using Air During Pentose Fermentation of Normally Toxic Lignocellulosic Prehydrolysates."The inventors are F. Keller and Q. Nguyen.

Outreach

Collins Pine/BCI PIER Project

(Contains sensitive information)

Two samples of lignin residues from softwood forest thinnings were produced for BCI for combustion characterization. The lignin samples were generated using a two-stage dilute acid hydrolysis method. Softwood forest thinnings from the Quincy, CA area were milled through 0.5-inch screen, impregnated with 0.8% sulfuric acid solution, then hydrolyzed at 185°C for 4 min in the 4-L NREL steam explosion reactor. The pretreated material was extensively washed with water, impregnated with 2.5% sulfuric acid solution, then hydrolyzed at 210°C for 2 min. The second-stage hydrolysate was divided into two equal portions and treated separately to generate the lignin samples. One hydrolysate portion was adjusted to pH 5 with calcium hydroxide, then fermented with *Saccharomyces cerevisiae* yeast. The fermentation slurry was filtered, extensively washed with water, then air-dried to approximately 83% solids. This sample was termed “fermented hydrolysate lignin residue.” The second hydrolysate portion was adjusted to pH 5 with sodium hydroxide, extensively washed, then air-dried to about 89% solids. The sample was termed “simulated enzyme hydrolyzed lignin residue.” The composition of washed second-stage hydrolysate was 21.7% cellulose, 0.7% hemicellulose, 74.8% lignin, 0.7% ash, and 2.1% unknown.

Cargill Dow CRADA Prehydrolysis Experiments

(Contains sensitive information)

Experimental procedures, analysis methods, and spreadsheet templates used to calculate the sugar yields and material balances of steam pretreatment using the 4-L steam gun have been transferred from NREL to Cargill Dow (CD).

A new material balance procedure was developed. This new procedure, which involves washing the entire pretreated sample instead of an aliquot as specified in the standard procedure, will provide comparison and validation of the standard procedure.

A draft plan has been developed to produce approximately 20 lb (oven-dry weight) of washed fiber from steam-exploded corn stover.

PROCESS ENGINEERING AND DEVELOPMENT

Summary of Technical Achievements or Results

The Process Engineering and Analysis team completed several important studies in this time period. These studies are important to the efforts of the program to understand the impacts of continued research on the cost of ethanol and other opportunities for biomass conversion.

- Completed the annual 20-year outlook for ethanol from biomass, which was a C milestone. NREL report pending publication.
- The MYTP was updated and published to aid in preparation of the FY2001 Annual Operating Plan.
- Completed the USDA/NREL joint project phase I work comparing starch and lignocellulose processes for ethanol production. This C milestone was published as an NREL report.
- Completed feedstock handling and solid/liquid separation investigations through the Harris Group subcontract.
- Organized and held a successful stakeholder meeting to kick off the Life Cycle Analysis (LCA) of corn stover. Participants represented the many aspects of LCA (environmental groups, feedstock growers, and product users).
- Completed technical memorandum reports on: 1) volatile organic compounds (VOC) emissions from process as predicted by the model and its implications to permitting a plant; 2) a combined ethanol-lignin process model to predict cost savings from a lignin fuel co-product; 3) development of a template for modeling biomass processes in Aspen; and 4) evaluation of labor costs for the biomass to ethanol process design.

General Technical and Scientific Progress

- Completed a C milestone with the finalization of the annual 20-year outlook for ethanol from lignocellulose, specifically corn stover. It will be published as an NREL report. This report details the possible improvements to the process through continued research and implementation of technologies envisioned for development. The outlook considers improvements from all sectors, including feedstock production and processing.
- The MYTP was published in August. For FY2001 and subsequent years, the Annual Operating Plan will be derived from the MYTP. This is a new goal, and is designed to improve the alignment of the annual operating plan (AOP) with the MYTP and ultimately with the DOE Biofuels program goals and plans.

- Phase I of the joint USDA/NREL project was completed. Working closely together, USDA-ARS and NREL engineers developed an understanding of the differences and possible synergies between the starch and lignocellulosic ethanol processes. An in-depth report (which was a C milestone) comparing the 2 processes was published as an NREL report. The models resulting from this phase will be used to create a combined process model to explore synergies of a co-location scenario.
- The subcontract with the Harris Group made substantial progress during this period, completing several key investigations. Feedstock handling for corn stover is one of three major areas of research for the Harris subcontract, and the final report detailing their findings and recommendations was completed. A draft report of the solid/liquid separation testing was provided and reviewed by NREL. Harris worked with Valmet (previously Sunds) to provide cost information for the go/no-go decision (a sector milestone) in the counter-current pretreatment project. Still underway is the reactor design and costing effort, which will provide comprehensive information about batch reactor design considerations and costs. The contract completion date is estimated to be 12/31/00. Results from this work will be used to update the process design and costing for the stover to ethanol process in February 2001.
- A LCA of corn stover to ethanol was initiated in May with a well-attended and highly successful kick-off meeting for stakeholders. A diverse group representing environmental, market, feedstock, and ethanol industry representatives provided vital input for the development of the analysis plan, which will look at the impacts of ethanol production and usage, from crop growth to end use in vehicles. Modeling of the entire life cycle of stover to ethanol will be completed in December 2000. The analysis will address sustainability issues in addition to the typical cradle to grave emissions study, and is an update of the first biomass to ethanol life cycle analysis performed in 1993.
- A fifth subcontract report from the Bridge to Corn Ethanol project was received from SWAN Biomass/Weatherly/High Plains and reviewed. A scope change for the final Bridge subcontract with Delta-T addresses different processes such as gasification and different feeds such as spent grain (or DDG) from dry mill plants. This change is an attempt to explore different options than the previous Bridge studies. The earlier Bridge studies identified a need for a more near term process while enzyme costs are being reduced via subcontracts with enzyme manufacturers. The potential of DDG as a near-term or interim feed has been discussed with corn ethanol producers, with mixed response. In addition to this work, several sensitivities performed by NREL investigating mixing corn and stover feed to an existing plant suggested that opportunities for cost savings do exist and should be more fully considered. Results of the Bridge subcontracts and NREL analyses were presented to the Renewable Fuels Association Board and at the OFD semi-annual review.
- Two different pretreatment processes were rigorously modeled using data from research members of the Consortium for Applied Fundamentals and Investigation (CAFI). Lime pretreatment (data provided by Texas A&M) and hot water pretreatment (data provided by Purdue) were evaluated using the NREL technoeconomic model. A third process model, ammonia explosion, is underway. The results will be reviewed

with the CAFI in November. The objective of this work is to evaluate different pretreatments in the biomass to ethanol process design and determine candidate processes to reduce the production cost. The base case for comparison is dilute acid hydrolysis.

- A project proposal for a 2-stage dilute acid process was completed and submitted to Merrick Engineers and Constructors in response to a request for technologies capable of converting softwood to ethanol in the near term. Merrick was contracted by SEAlaska to compare several processes and recommend the most viable one. The design for 275 TPD was tailored to a site co-located next to SEAlaska's veneer mill.
- A simple tool was developed that explores the costs of ethanol production. Data from the rigorous process model was regressed to provide a simplified set of equations for the tool. The tool is accessible from the Biofuels Web site.
- A considerable effort by the engineering team in collecting and compiling information on renewable diesel processes provided the basis for the Biodiesel project's P milestone to evaluate the renewable diesel program.
- A continued effort between the engineering team and EPD team to investigate corn stover composition data provided valuable information on variations and the possible causes. Different analytical and reporting methods must be considered when using data collected from more than one study; however, the value of using multiple sources is that they can provide an average in regional, seasonal, and varietal differences.
- In this period, the engineering team began providing design and modeling support to Cargill-Dow, starting with a comprehensive literature search on pretreatment methods that fit a well-defined set of guidelines. The best of these will be used to evaluate process designs for producing sugar from fiber and stover to feed Cargill-Dow's polylactic acid process. We have begun to develop a base case model that will be used to evaluate the pretreatment options.
- Performed pre-feasibility studies of black poplar for a Spanish engineering firm, INCIR, in Zaragoza, Spain, and rice straw for an Arkansas farmers' LLC. in support of the Partnership Development team.
- Presented information on the cost of fermentable sugars at the Green Chemistry Conference in June 2000. An outline of the enzymatic biomass to ethanol technology and the required transfer prices (both base case and out year) of a dilute sugar stream were presented. This work is intended to reach companies interested in other products from biomass sugars. Such an approach can help commercialize the ethanol industry by proving and commercializing the production of sugars first, after which ethanol production is the next step, with less risk than the entire process at once.
- Completed a comprehensive review of the mechanism for registering fuel components with the EPA through a subcontract with J.E. Sinor Consultants. This review provides information to the lignin improvement project about the steps and level of data required

to gain approval of a lignin derived octane enhancer. In addition, during this period, an NREL report entitled "Review of Market for Octane Enhancers," was published. The work was conducted under a subcontract with J.E. Sinor Consulting in the first half of FY00 and details the potential market for a lignin derived octane enhancer.

Scientific Publications, Presentations, and Other Activities

Ruth, M., "The Potential Cost of Lignocellulosic Sugar for Commodity Chemical Production," presented at the Green Chemicals Conference, June, 2000.

Ibsen, K., McAloon, A., Wooley, R., Taylor, F., Yee, W., "Comparing the Cost of Producing Ethanol from Starch and Lignocellulosic Feedstocks," NREL/TP-580-28893, October, 2000.

Ruth, M., Ibsen, K., Sheehan, J., "Twenty Year Outlook for the Cost of Ethanol from Lignocellulosic Biomass Produced by the Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis Process," June, 2000.

Sheehan, J., "Bioethanol Multi-Year Technical Plan," August, 2000.

Ibsen, K., "Building a Bridge to the Corn Ethanol Industry," presentation at the DOE Semi-Annual Management Review, July 2000.

Aden, A., Nguyen, Q., Yancey, M., "Wood Waste to Ethanol; SEAlaska Project Proposal," presented to Merrick & Company, April, 2000.

Sinor, J., "Review of Market for Octane Enhancers," NREL/SR-580-28193, May, 2000.

RENEWABLE DIESEL PROJECT

Summary of Technical Achievements or Results

On the behalf of industry, the National Biodiesel Board (NBB) submitted the Tier II Health Effects report to EPA and held various press events to celebrate. No discernable impacts were noted for 1x and 5x concentrations of biodiesel exhaust on animal populations—on which over 20 major organic functions were measured. At the 10x level, minor changes in some organic functions were measured, but these faded once exposure ceased. NREL co-funded this work with NBB and the soy industry. Biodiesel is the only alternative fuel to have met this milestone to date.

NREL is working on an evaluation of renewable diesel fuels to identify new research and development opportunities for OFD. Biogas for natural gas vehicles, dimethyl ether (DME) for propane vehicles, Fischer Tropsch, and alternative fuel-diesel blends show promise. The report should be finished by the end of the calendar year and follow-on work to better define promising options is under consideration.

Final comments were provided to the Institute of Gas Technology (IGT) on the Multifeedstock Study draft report. Once a completed version has been received, NREL will publish a series of reports on the entire project which will include B100 and B20 emissions and ignition characteristics, B100 and B20 fuel properties, properties of fatty acid fuel constituents, and bioassay results of various types of B100s.

Environ has prepared biodiesel emission databases for use in EPA and the California Air Resources Board (CARB) air shed models but the project is on hold until CARB issues its decision on what types of data they want to use in future modeling exercises. Evaluations of ozone in Chicago and the Northeast Corridor are underway, as is an evaluation of CO levels in Las Vegas. By conducting this work, we hope to show that biodiesel can be a tool for air quality planners.

General Technical/Scientific Progress

Collaborative Activities

USDA Philadelphia has produced seven faux biodiesel fuels for testing. NREL will test the fuels at Williams Lab to determine which, if any, meet ASTM standards for diesel and biodiesel. Three of the seven will be integrated into NREL's NO_x reduction testing program at CSM this fall. In the same program, USDA Peoria also wishes to test four additives that may reduce NO_x. A testing contract with CSM will be signed in October. Data for CARB on CARB/B20 blends are also included. The project also includes a cooperative task with the Office of Heavy Vehicle Technology to test blends of Fischer-Tropsch and biodiesel fuels.

NREL has debriefed World Energy on its CARB work to date. World Energy will establish further regulatory support for biodiesel in CA through legislation. They are responsible for

converting a fleet of 94 recycling and garbage trucks to B100 in San Jose, CA. B100 is not a legal fuel in CA.

Ocean Air Environmental Fuels and Glycerin (formerly NOPEC) will work with NREL to establish a consortium of trap grease collectors and several representatives of local municipal water treatment organizations who are interested in a demonstration project for trap grease biodiesel production. NREL will be developing a plan for a feasibility study with the group in the fall and has reserved funds to cost-share the feasibility analysis.

CSX railroad has loaned NREL a locomotive for emission testing at SwRI. The American Railroad Association and CARB are interested in the outcome of the project. Fuel has been purchased and tested. Two B20 tests and two baseline tests will begin in late October for CARB/B20 and EPA locomotive test fuel/B20.

NREL is also working with the University of Puerto Rico, Mayaguez and the Puerto Rico Energy Office to build biodiesel expertise in the Caribbean. The Puerto Rico project will expand markets for biodiesel through demonstrations, evaluate process improvements for low cost recycled grease biodiesel, and document oil displacement benefits in Puerto Rico.

NREL is working with the Regional Biomass Energy Program (RBEP) on a variety of areas. NREL and Biodiesel Development Corporation (BDC) are providing technical guidance for a Western Regional Biomass Energy Program (WRBEP) biodiesel project in NV and CA. The WRBEP project will conduct resource assessment in CA and NV and evaluate processes that reduce water consumption and expand potential sites for biodiesel.

In FY2001, NREL, the local energy offices, and state organizations plan to hold one biodiesel workshop in each RBEP region. A contractor will provide logistical support.

A grass roots educational effort was initiated with environmental groups through a contractor. The contractor will build a constituency for biodiesel in the environmental community in order to provide a more diverse viewpoint on the benefits and costs of biodiesel. The results will be shared with the RBEPs and NBB.

NREL issued a solicitation to provide technical support to the Green Parks Program for biodiesel. No acceptable bids were received and the project was cancelled by the mutual consent of J. James and S. Tyson.

Several representatives from AAE, a firm promoting the use of ethanol-diesel fuel blends, met at NREL in July to discuss further demonstration needs. S. Tyson will work with AAE to determine if there is any interest at DOE to support demonstration projects.

University of Toronto has announced its patent-pending technology for a one-step conversion process for high-free fatty acid feedstock. NREL performed some limited testing on the final products. A final ASTM test has not been performed and NREL cannot substantiate the University of Toronto's claims at this time. BDC may test a scaled-up version of this technology in its portable PDU this fall. Some output may be provided to NREL for testing, and further research and development needs could be identified at that time.

NREL withdrew a proposal to WestStart-CALStart and the U.S. Army TACOM division on a recycled grease biodiesel demonstration project. TACOM has made progress on a project that duplicates the proposal and it is unclear at this time if further R&D is needed.

The final phase of the Oxidative and Thermal Stability Test Methodology got back on track after resolving some confusion over fuel samples. The NBB supported repeated testing of the samples. The Illinois Soybean Board and Chevron will support the mini round robin testing this spring.

NREL and our co-sponsor, NBB, have started planning the fourth Annual Biodiesel Brainstorming Coordination Meeting. Ocean Air and World Energy has agreed to co-host the meeting in or near Lakeland FL in the spring of 2001. NBB will provide a mailing list for invitations and develop a speaker list with NREL. NREL will make all the logistical arrangements.

NREL and the Fats and Protein Research Foundation (FPRF) will jointly work on a Life Cycle Analysis of Yellow Grease Biodiesel Production. FPRF will provide industry data and NREL will support modeling and report development in FY 2000 and 2001.

Brookhaven National Laboratories, NBB, and FPRF are developing an research and development project with NREL and New York State Energy Research and Development Authority (NYSERDA) to test biodiesel and greases as heating oil fuels in a variety of boilers. Emission test data for regulators will be a major outcome and remove a hurdle to permitting the use of these fuels in existing boiler systems. Recommendations for modifications will also be provided. This project was initiated due to heightened interest in biodiesel and oils for use as emergency fuels in the Northeast.

Other collaborative activities include:

- S. Tyson attended NBB meetings in June and September 2000. Feedstock neutrality was a major topic. The industry appears to be fighting over markets that are substantially smaller than their combined capacity. As a result, biodiesel prices (delivered to customers) have dropped to between \$1.25 and \$1.80 per gallon. Dan Reicher, Assistant Secretary of Energy Efficiency and Renewable Energy (EERE) presented and held discussions with industry participants. Broader inclusion of biodiesel in Office of Transportation Technologies (OTT) and the Office of Power Technology (OPT) programs was a message that the NBB brought home. NBB complaints to DOE clearly suggest that the use of taxpayer funds for biodiesel purchases is not an acceptable topic for public policy discussions.
- NREL representatives also attended a WRBEP meeting in Salt Lake City, UT, in March 2000 for proposal review and selection. A meeting at BioEnergy 2000 to discuss AOPs and requests for proposals (RFPs) for 2001 was planned for October, 2000 in Buffalo, NY.

Scientific Publications, Presentations and, Other Activities

In March 2000, S. Tyson met with Andrew Gray, Comptroller's Office, DOE, to review the Biodiesel Program's goals and accomplishments.

In June, 2000, S. Tyson chaired a session on alternative fuels at the Windsor Workshop 2000 in Toronto Canada.

In July, 2000, the DOE/OFD Semi Annual Update and the Biodiesel Update were completed.

In September, 2000. S. Tyson and S. Howell of the NBB met with Tom Gross and other OTT office staff to expand R&D programs to include more B20 and B100 test elements.

Technology Transfer

Four thousand copies of the biodiesel Fact Sheet "The Clean Green Fuel for Diesel Engines", have been distributed and another reprint was ordered. Biodiesel producers have found it very useful in their educational outreach activities.

S. Tyson appeared with the NBB and various biodiesel producers and customers on a short segment about biodiesel that was broadcast on the CBS Today Show.

S. Tyson and Proforma visited three biodiesel producers to review U.S. biodiesel technology. Proforma is constructing engineering and economic analysis of various biodiesel processes and scales. This work should identify where process improvements can make significant impacts on costs.

Project Operations

Biodiesel R&D Targets: Feedstock (Phase 1) - A hybrid mustard breeding contract with the University of Idaho has been modified to expand the focus on pesticide varieties and their impacts on farm systems. Crop rotation impact on wheat, and some laboratory testing of pesticide strength was included. A literature review is due in September, 2000 and a marketing study is planned for FY 2001. In addition, an annual peer review panel to provide technical support and guidance is in the planning stages.

Colorado School of Mines continues work on the fundamental combustion of biodiesel with respect to NO_x emissions. Equipment failures have created some delays, but progress has continued.

The Waste Grease Composition Analysis with industrial partner Pacific Biodiesel (PB) is being renegotiated at this time due to poor performance. All options, including rebidding the work are under consideration.

The legal office has not resolved the removal of the biodiesel tanks from Chicago yet. The issue is still under investigation.

The “virgin” and “soy” biodiesel law suits were dismissed and no future action is underway at this time. General Services Administration (GSA) guidance on virgin and soy oil is still a problem, but DOE has been working with GSA to modify the guidance to be less rigid. World Energy is also working with GSA to modify the guidance at NREL’s request.

Public relations work for “Petroleum Infrastructure Development,” was competed for \$40,000 and initial work by the contractor has been very good. Several more petroleum firms now market B20 and the interest is very high in other markets. Some firms have requested educational materials, which NREL provided. The contractor will make several appearances at local and national conferences in the petroleum sector to expand the audience and maximize value of the remaining funds.

Negotiated changes to “Oil Crop Potential for Biodiesel Production,” with University of Idaho XCO-9-29095-01 and added remaining funds for FY 2000.

Modification of “The Impact of Biodiesel Fuels on Air Quality and Human Health,” with Environ International Corp, AXE-9-29079-01. The remaining \$68,790 will be funded in FY 2000.

REPORT DOCUMENTATION PAGE			<i>Form Approved</i> OMB NO. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE December 2000		3. REPORT TYPE AND DATES COVERED Management Report FY 2000
4. TITLE AND SUBTITLE Biofuels Program Semiannual Report				5. FUNDING NUMBERS BFP1A101
6. AUTHOR(S) R. Wooley				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)				8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) National Renewable Energy Laboratory 1617 Cole Blvd. Golden, CO 80401-3393				10. SPONSORING/MONITORING AGENCY REPORT NUMBER NREL/MP-580-29296
11. SUPPLEMENTARY NOTES NREL Technical Monitor:				
12a. DISTRIBUTION/AVAILABILITY STATEMENT National Technical Information Service U.S. Department of Commerce 5285 Port Royal Road Springfield, VA 22161				12b. DISTRIBUTION CODE
13. ABSTRACT (<i>Maximum 200 words</i>) Management report for the second half of fiscal year 2000. The report summarizes the achievements and activities of the Pretreatment Technology, Improved Cellulase Enzymes, Strain Development, Enzymatic Process Development, Lignin Conversion to Fuels, Collaborative Industrial Process Development, Process Engineering and Development, and Renewable Diesel Project teams.				
14. SUBJECT TERMS Biofuels management, FY 2000, Pretreatment, Cellulase Enzymes, Strain Development, Enzymatic Process, Lignin Conversion, Renewable Diesel				15. NUMBER OF PAGES 45
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
20. LIMITATION OF ABSTRACT UL				